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PRINCIPAL INVESTIGATOR: Waun Ki Hong, M.D.
Roy Herbst, M.D., Ph.D.
Li Mao, M.D.
Edward Kim, M.D.

CONTRACTING ORGANIZATION: The University of Texas
M.D. Anderson Cancer Center
Houston, TX 77030

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6. AUTHOR(S)
Waun Ki Hong, M.D.
Roy Herbst, M.D., Ph.D.
Li Mao, M.D.
Edward Kim, M.D.
E-Mail:

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M.D. Anderson Cancer Center
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14. ABSTRACT

The Program BATTLE seeks to establish individualized targeted therapy by prospectively examining patients' tumor biomarker profiles and assigning them to corresponding targeted therapies with the expectation to yield a better clinical outcome. Based on common altered signaling pathways in lung cancer, the BATTLE Program proposes to develop four phase II trials for chemorefractory, advanced NSCLC patients: erlotinib, ZD6474, bexarotene with erlotinib, and sorafenib which target, respectively, EGFR, VEGF / VEGFR, retinoid X receptor and cyclin D1, and Ras / Raf signaling pathways. A novel adaptive randomization statistical design will be applied to the clinical trials to accelerate the identification of best-fit treatment for patients. We propose also to study the molecular mechanisms of response or resistance to these targeted agents, identify novel molecular features in tumors and surrogate tissues to correlate with tumor response or resistance to the agents and, finally, explore other novel targeted agents (RAD001 and perifosine) in combination and their mechanisms of action by targeting mTOR and PI3K/Akt signaling, and develop phase

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INTRODUCTION

Lung cancer is the leading cause of cancer-related death in both men and women in the United States. Chemotherapy has reached its limit in improving the survival of lung cancer patients. Therefore, a different strategy must be waged in the battle against lung cancer. Targeted therapy, a newly emerged therapeutic approach in lung cancer, has succeeded in some cancer types and demonstrated its initial success in the treatment of lung cancer when a class of targeted agents termed epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors, such as gefitinib and erlotinib, improved tumor response rates in patients with advanced non-small cell lung cancer (NSCLC), which was strongly correlated to the presence of *EGFR* mutations in the tumors (Cappuzzo and Hirsch et al., 2004; Cappuzzo and Magrini et al., 2004; Gatzemeier et al., 2004; Herbst and Giaccone et al., 2004; Herbst and Prager et al., 2004; Herbst and Sandler et al., 2004; Lynch et al., 2004; Kobayashi et al., 2005; Miller et al., 2004; Pao et al., 2004; Paez et al., 2004; Shepherd et al., 2004; Shigematsu et al., 2005). This has for the first time demonstrated the importance of selecting patients for individualized targeted therapy in NSCLC.

The Program **BATTLE** (**B**iomarker-integrated **A**pproaches of **T**argeted **T**herapy for **L**ung Cancer **E**limination) seeks to establish individualized targeted therapy by prospectively examining patients' tumor biomarker profiles and assigning them to corresponding targeted therapies with the expectation to yield a better clinical outcome. This novel approach will be a proof-of-principle experiment to test the benefit of molecular-based individualized targeted therapy for lung cancer patients. Specifically, the objectives of the BATTLE program are:

1) To establish a clinical trial program using biomarkers to select individualized targeted therapy for patients with chemorefractory advanced NSCLC through the implementation of molecular classification based on the status of specific targeted biomarkers and adaptive randomization via hierarchical Bayes modeling.
2) To study the molecular mechanisms of response and resistance to targeted agents to discover new signaling pathways for test in future trials.
3) To identify molecular features in tumor tissues to correlate with tumor response or resistance, and identify serum biomarkers as surrogates.
4) To investigate other targeted agents in combination to overcome the resistance due to novel signaling pathways (e.g., mTOR and PI3K/Akt) and improve treatment efficacy.

BATTLE is composed of four Specific Aims with four phase II clinical trials and an umbrella protocol in Aim 1, six research projects in Aims 2 - 4, and two potential phase I trials in Aim 4. Here, we present our scientific progress of the BATTLE program for the second grant year.

PROGRESS REPORT

Aim 1 To establish a clinical trial program using biomarker assessment to select individualized targeted therapy for previously treated chemorefractory advanced NSCLC patients.

(PI, Co-PIs, and Investigators: Drs. Waun Ki Hong, Roy Herbst, Edward S. Kim, George Blumenschein, Anne Tsao, Hai Tran, Marshall Hicks, Rodolfo Morice, Bruce Johnson)

Specific Aim 1 has five clinical trials: one umbrella trial and four Phase II open-label trials. After screening, eligible patients are enrolled in the umbrella trial, and tumor biopsies are taken for biomarker analysis conducted by the Biomarker Core. (For details, please see the Biomarker

Core section of this report.) Biomarker results are analyzed by the Biostatistics and Data Management Core. (For details, please see the Biostatistics and Data Management Core section of this report.) There are two components of this study: 1) an equal randomization phase, where patients are randomized equally to the four trials after biomarker analysis; and 2) an adaptive randomization phase, where patients are enrolled to one of the four clinical trials based on their tumor biomarker characteristics. The four Phase II clinical trials are presented in the four sub-aims of Aim 1 described below and depicted in Figure 1. An update is provided following the list of subaims.

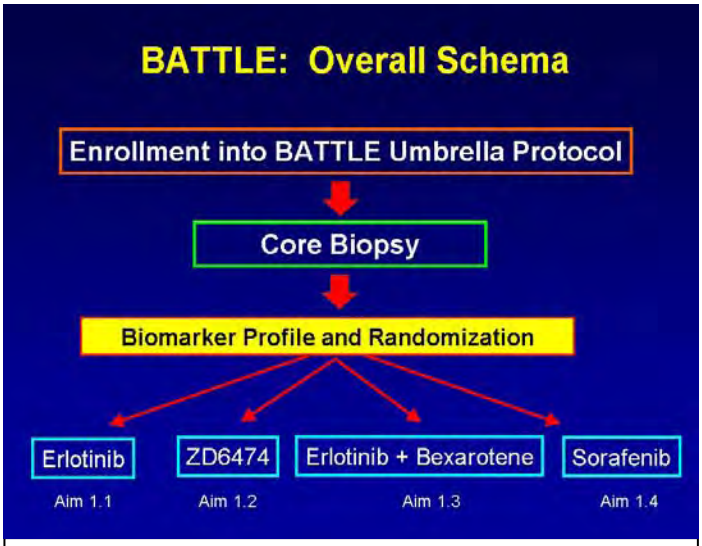


Figure 1. Overall Schema for BATTLE Trials.

Aim 1.1 To conduct a clinical trial with erlotinib in patients with previously treated advanced NSCLC whose tumors have EGFR mutations and / or overrepresentation.

Primary objective is to determine the 8-week progression-free survival (PFS) rate of patients with previously treated advanced NSCLC whose tumors have EGFR mutations and / or overrepresentation who are treated with erlotinib.

Secondary objectives are to 1) determine the overall survival rate, response rate, and toxicity profiles of patients with advanced NSCLC whose tumors have EGFR mutations and / or overrepresentation and treated with erlotinib, 2) determine the plasma and (if available) tumor tissue concentrations of erlotinib and their correlation with response and toxicity by using pharmacokinetics and pharmacodynamic modeling.

Aim 1.2 To conduct a clinical trial with ZD6474 in patients with previously treated advanced NSCLC whose tumors have increased VEGF and / or VEGFR-2.

Primary objective is to determine the 8-week PFS rate in patients with previously treated advanced NSCLC whose tumors have increased VEGF and / or VEGFR-2 who are treated with ZD6474.

Secondary objectives are to 1) determine the overall survival rate, response rates, and toxicity profiles of patients with advanced NSCLC whose tumors express increased VEGF and / or VEGFR-2 and treated with ZD6474, and 2) determine the plasma and (if available) tumor tissue levels of ZD6474 and their correlations with response and toxicity by using pharmacokinetics and pharmacodynamic modeling.

Aim 1.3 To conduct a clinical trial with the combination of bexarotene and erlotinib trial in patients with previously treated advanced NSCLC whose tumors have expressed RXRs and / or increased cyclin D1.

Primary objective is to determine the 8-week PFS rate in patients with previously treated advanced NSCLC whose tumors have expressed RXRs and / or increased cyclin D1 who are treated with the combination of Bexarotene and Erlotinib.

Secondary objectives are to 1) determine the overall survival rate, response rate, and toxicity profiles of patients with advanced NSCLC whose tumors have expressed RXRs and / or increased cyclin D1 and treated with the combination of bexarotene and erlotinib, 2) determine the plasma and (if available) tumor tissue concentrations of bexarotene and erlotinib and their correlation with response and toxicity by using pharmacokinetics and pharmacodynamic modeling.

Aim 1.4 To conduct a clinical trial with sorafenib trial in patients with previously treated advanced NSCLC whose tumors have mutated *K-ras* and / or *B-raf.*

Primary objective is to determine the 8-week PFS rate in patients with previously treated advanced NSCLC whose tumors have mutant *K-ras* and / or *B-raf* who are treated with sorafenib.

Secondary objectives are to 1) determine the overall survival rate, response rate, and toxicity profiles of patients with advanced NSCLC whose tumors have mutated K-ras and / or B-raf and treated with sorafenib, 2) determine the plasma and (if available) tumor tissue concentrations of sorafenib and their correlation with response and toxicity by using pharmacokinetics and pharmacodynamic modeling.

Update

Considering the highly interactive nature of the clinical trials in the BATTLE program, we will report the progress of the all the clinical trials in an integrated way.

The five BATTLE clinical protocols went through an extensive and comprehensive process of the protocol review, revision and approval by M. D. Anderson Cancer Center (MDACC) Clinical Research Committee (CRC), MDACC Institutional Research Board (IRB), the Department of Defense (DoD), Food and Drug Administration (FDA), and four pharmaceutical companies. On November 27, 2006, the protocols were activated for patient enrollment, less than eight months after the BATTLE grant was activated (April 1, 2006). This timeline is the shortest for protocol review, approval, and activation in our Department for a large multidisciplinary, integrated multi-trial program. This rapid activation can be attributed to our departmental effort and, specifically, due to the dedication of our medical oncologists, protocol coordinator, research nurses, Biostatistician Core, Biomarker Core, and our institutional regulatory personnel.

Since activation, patient accrual has been excellent (Table 1). Response to our medical oncologists, interventional radiologists, and research nurses introduction of the BATTLE trials has been overwhelmingly positive. This program has become a model within our institution for personalized cancer therapy. In addition, patients are highly interested and inquiring independently regarding the program. The coordination between the surgeons, medical oncologists, Biomarker Core and Biostatistics Core, has been well-planned and the clinical trials have been running very smoothly.

Biomarker results have been obtained in 120 of 145 (84%) of patients (see Biomarker Core section for details). Clinical data and tissue specimens from the patients have been and

continue to be collected for patient stratification into specific trials. Some of these tissue specimens have been distributed (and will continue to be as planned) to support research projects in the BATTLE grant, specifically for biomarker discovery aspects.

By March 31, 2008, 160 patients were registered, 150 biospies performed, with a total of 114 patients treated to date, 97 patients equally randomized, and 17 patients adaptively randomized (Table 1).

Table 1. Accrual to BATTLE Trials (11/27/06 to 03/31/08).

	No. of Patients
BATTLE Umbrella Protocol, consented	160
Total number of biopsies	150
Total Patients Treated	114
Patients Equally Randomized to 4 trials	97
Patients Adaptively Randomized to 4 trials	17

Enrollment is ongoing. No significant adverse events have been reported for these treatment studies at this time.

The patient accrual has been brisk. Additionally, the first 62 or 72 (86%) patients' baseline serum characteristics were analyzed and are scheduled to be presented at the 2008 ASCO Meeting (see Aim 2.3, objective 3 for details). Discovery biomarker experiments are ongoing in collaboration with the PI's of the basic and translational research aims as described throughout this report. The BATTLE program was highlighted in our *Science Day: Lung Cancer Research* symposium, March 26, 2008, with almost 200 attendees and in our External Advisory Board Meeting the following day (see Appendix B).

The BATTLE trials are currently going through the regulatory process at Dana-Farber Cancer Institute (DFCI) and we plan a site visit and initiation in late summer. The plan was to open these trials at DFCI once the adaptive randomization phase was activated.

Key Research Accomplishments:

- Activated all five BATTLE clinical protocols at M. D. Anderson Cancer Center within the first grant year.
- 166 patients registered and 119 randomized into one of the four treatment arms.
- Adaptive randomization phase accruing.
- Patient accrual and interest continue at a healthy pace.
- The success rate of quality tissue specimen acquisition and biomarker evaluation is over ~84%.
- Demonstrated highly efficient collaboration of Clinical teams, Biostatistics Core, and Biomarker Core.
- Developed the largest translational research program requiring core biopsy samples ever run in our department and, possibly, in the country.

Reportable Outcomes:

Presentations

- Herbst R, Lee JJ. The BATTLE Project. Presentation to the M. D. Anderson National Cancer Institute Cancer Center Support Grant External Advisory Board. Houston, Texas; January 2007.
- Institute of Personalized Cancer Therapy Retreat, The University of Texas M. D. Anderson Cancer Center, Houston, TX. February 2008 (Herbst)

Abstracts

In collaboration with Biostatistics and Data Management Core:

- Zhou X, Kim ES, Herbst RS, Liu S, Wistuba II, Mao L, Lewis J, Lippman SM, Hong WK, Lee JJ. A clinical trial design applying Bayesian adaptive randomization for targeted therapy development in lung cancer - A step toward personalized medicine. Submitted to American Society of Clinical Oncology (ASCO) Annual Meeting, Atlanta, Georgia; June 2007.
- Liu S, Kim ES, Zhou X, Wistuba II, Herbst RS, Lewis J, Lee JJ. An Application of Adaptive Randomization Using Hierarchical Bayes Model in a Prospective Biomarker-Based Clinical Trial. Submitted to the Joint Statistical Meeting, Salt Lake City, Utah; August 2007.

In collaboration with PIs of Aims 2.3 and 3 and Biomarker and Biostatistics Cores:

- Tran HT, Kim ES, Lee JJ, Herbst RS, Liu S, Wistuba II, Yan S, Stewart DS, Hong WK, Heymach JV. Correlation between plasma cytokine/angiogenic factors (C/AF) and pathway activation from baseline tumor biopsy specimens in patients with advanced non small cell lung cancer (NSCLC): preliminary analysis from the Biomarker-based Approaches of Targeted Therapy for Lung Cancer Elimination (BATTLE) clinical study. AACR Annual Meeting, San Diego, CA, April 2008.

Publications (In Press)

In collaboration with Biostatistics and Data Management Core:

- Zhou X, Liu S, Kim ES, Herbst RS, Lee JJ. Bayesian Adaptive Design for Targeted Therapy Development in Lung Cancer - A Step Toward Personalized Medicine. In press, *Clinical Trials*, 2008.

Appendices:

Appendix B: Science Day Binder and External Advisory Board Agenda

Conclusions:

The completion of the clinical trials is the key to this BATTLE research program. In the first grant year, the program was significantly ahead of our proposed timeline. This has continued after the first year of activation and, in addition to the trial performance, we have supported the early efforts in biomarker discovery in collaboration with the other projects. The trial accrual is reflective of the goals of the department and the program in its completion. The design and innovative nature of the trials will keep interest high among patients who are treated at M. D. Anderson. Accrual is ongoing and will help support the other BATTLE specific aims with tumor response data, tissue specimens, and biomarker information.

Specific Aim 2: To investigate molecular mechanisms of response and resistance to the targeted agents used in the BATTLE program.

Specific Aim 2.1. To validate the molecular mechanisms of response and resistance to erlotinib for patients with chemorefractory NSCLC.

(PI and Co-PI: Bruce Johnson, M.D., and Pasi Jänne, M.D., Ph.D.)

The association between somatic epidermal growth factor receptor (*EGFR*) mutations and clinical response to gefitinib in patients with non-small cell lung cancer (NSCLC) was published in 2004. This proposal builds on previous findings to further characterize *EGFR* mutations in subjects' tumors and in tumor cell lines and the relationship of these mutations, subject outcome, and *in vitro* behavior to different EGFR inhibitors. The data generated demonstrates that subjects whose NSCLCs have *EGFR* mutations typically respond to single-agent therapy with gefitinib, are treated for a median of 1 year or longer, and achieve a median overall survival duration longer than 2 years. This survival duration is 3-fold longer than that achieved with conventional chemotherapy in previously untreated subjects with NSCLC. The patients treated with gefitinib or erlotinib with increased copy number assessed by fluorescence in situ hybridization (FISH) have a response rate of 20-30% and the patients live a median of approximately 2 years. The goal of this research is to confirm these initial observations in prospective cohorts of subjects with NSCLC and somatic *EGFR* mutations or increased copy number with erlotinib as the initial therapy. This proposal is generating translational information on somatic mutations and copy number, prospective validation of the outcome of patients with NSCLC and *EGFR* mutations or increased copy number treated with erlotinib, information on activation of the EGFR pathway in NSCLC and NSCLC cell lines, and information about mechanisms of resistance.

Objective 1: Establish estimates of the response and outcome of previously treated patients with prospectively identified somatic *EGFR* mutations treated with erlotinib.

There have been 35 patients accrued to the trial entitled, "A Phase II, Open Label Study of Erlotinib (Tarceva®) in Previously Treated Subjects with Advanced Non-Small Cell Lung Cancer." The analyses for EGFR mutations, copy number, and immunohistochemistry are being done by the Biomarkers Core to correlate the laboratory findings with the outcome.

Objective 2: Determine effects of TGF-α, EGF, and AR on the growth of *EGFR* mutant and wild type cell lines.

Update

Methods:

Cell Lines and Drugs. The following cell lines were used for objectives 2 to 4: The 14 NSCLC cell lines (PC9, HCC827, HCC4006, H3255, Calu3, H1648, H1437, HCC15, HCC193, HCC95, H661, H2126, H1666, H358) and 4 head and neck cancer cell lines (HN11, HN12, HN13 and HN28) have all been previously characterized. All cells except H3255, H1666 and H1648 were cultured in RPMI-1640 (Sigma Chemical Co., St Louis, MO) supplemented with 5 or 10% fetal bovine serum, 100 U/ml streptomycin and 1 mM sodium pyruvate. The H3255, H1666 and H1648 cells were cultured in ACL-4 media (Invitrogen, Rockville, MD) supplemented with 5% fetal bovine serum, 100 U/ml streptomycin and 1 mM sodium pyruvate.

Gefitinib was obtained from commercial sources and was purified through an ethyl acetate extraction. The resulting product was verified by liquid chromatography and mass spectrometry. Stock solutions of 10 mM were prepared in DMSO and stored at –20 ^{o}C. Cetuximab (2 mg/mL) was purchased from the pharmacy at Dana-Farber Cancer Institute and stored at 4^{o}C. The drugs were diluted in fresh media prior to use.

Detection of Ligands. EGF, amphiregulin, and TGF-α were measured in cell culture media using enzyme-linked immunosorbent assays (ELISA). These were performed according to the manufacturer's recommended procedures (Quantikine ^{TM}, R&D Systems, Minneapolis, MN) and as previously described. Briefly, confluent cells grown in serum containing media were washed with PBS and cultured in serum-free medium for 24 h. The cells were then incubated in fresh serum-free media for another 48 h, the media was collected and centrifuged, and the supernatant was stored at –70°C until analysis. All samples were run in triplicate. Color intensity was measured at 450 nm using a spectrophotometric plate reader. Growth factor concentrations were determined by comparison with standard curves.

Real-time reverse transcription-PCR (RT-PCR) analysis. Total RNA was isolated from NSCLC and HNSCC cell lines using Trizol (Life Technologies, Gaithersburg, MD) according to the manufacturer's specifications. One microgram (1 μg) of total RNA was primed with oligo(dT)$_{20}$ (Invitrogen, Carlsbad, CA), and cDNAs were synthesized with Transcriptor Reverse Transcriptase (Roche Applied Sciences, Indianapolis, IN) according to manufacturer's specifications. For endogenous control selection, equal volumes of cDNAs were analyzed using the TaqMan Human Endogenous Control Plate (Applied Biosystems, Foster City, CA) according to manufacturer's specifications. Human phospo glycerol kinase 1 (PGK1) was selected as endogenous control as its level of expression showed least standard deviation (0.79) across all samples (data not shown). The cDNAs were then used for real time PCR using TaqMan chemistries for human PGK1 (Part No. 4333765) and amphiregulin (Assay ID Hs00155832_m1). Levels of amphiregulin expression relative to HCC4006 were determined using the ddCt method and ABI 7500 Fast System SDS Software.

Cell proliferation and growth assays. Growth inhibition was assessed by MTS (3-(4, 5-dimethylthiazole-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphonyl) 2H-tetrazolium, inner salt) assay (Promega, Madison, WI)). This study was performed as in our prior studies in a 96-well format. The number of cells for each cell line required to obtain an optical density (OD) of 1.3 – 2.2 at 490 nm, the linear range of the assay, after 6 days of growth was determined empirically. The number of cells per well used in this study were as follows: HN28 1500; HN11, H1666, 2000; HN12, H1648, 3000; H358, HN13, HCC95, 4000; H1437, HCC193, H2126, 5000; Calu3, H661, 7000. After 24 hours, medium was replaced with RPMI-1640 containing 0.1% fetal bovine serum with or without drug. Gefinitib was used at concentrations ranging from 3.3 nM to 10 μM and cetuximab at concentrations ranging from 33 ng/ml to 100 μg/ml, similar to amounts used in prior reports by us and others. The amphiregulin antibody was purchased from R&D systems (AF262; R&D Systems, Minneapolis, MN) and used in cell proliferation assays as previously described. The cells were incubated for 6 days in the presence of drug. All experimental points were set up in six to twelve wells and all experiments were repeated at least three times.

Results:

Amphiregulin expression in medium. We first analyzed the concentrations of EGFR ligands, amphiregulin, EGF and TGF-α in the cell culture media of fourteen NSCLC and four HNSCC cell lines using an ELISA assay. Four NSCLC cell lines contained *EGFR* mutations (E746_A750del in PC-9, HCC827 cells, L747_E749del in HCC4006 cells, L858R in H3255 cells), while the

remaining cells were *EGFR* wild type. All except H358 were also *KRAS* wild type. Although amphiregulin concentrations varied from undetectable to 85 pM, it was detected in the majority of *EGFR* wild type cell lines while the 4 *EGFR* mutant cell lines produced undetectable (n=1) or very low (n=3) levels of amphiregulin (Figure 2). Only 2 cell lines, H1666 and Calu-3, produced any detectable amount of TGF-α while it was undetected in the remaining cell lines. We have previously demonstrated that H1666 produces TGF-α (Paez et al, 2004). EGF production was detected at very low levels in only 6 cell lines (HN11, H1666, HN12, H358, HN13 and H661). We did not detect TGF-α or EGF in the supernatant of any of the *EGFR* mutant cell lines.

Exogenous amphiregulin added to concentrations of ≥15-20 pM can stimulate the proliferation of murine keratinocytes and the GEO colon cancer cell line. Based on these findings we chose 20 pM as the minimum biologically significant concentration of amphiregulin. We divided the *EGFR* wild type cell lines into 2 categories: high amphiregulin group (n=7; HN11, H1666, HN12, H358, Calu3, HN13 and H1648) in which we detected ≥ 20 pM amphiregulin and low amphiregulin group (n=7; H1437, HCC15, HCC193, HCC95, H661, HN28 and H2126) in which we detected < 20 pM of amphiregulin in the cell culture media. We next used quantitative PCR (QPCR) to examine for any differences in amphiregulin mRNA produced by these cell lines. We compared the level of amphiregulin expression in the different cell lines to that in HCC4006 as this cell line produced nearly undetectable levels of amphiregulin as detected by ELISA (Figure 2). We detected significantly higher levels ($p < 0.05$; paired t-test) of amphiregulin mRNA in the high amphiregulin producing cell lines defined by ELISA compared to the low amphiregulin group. However, there was not a 100% correlation as in some cell lines (for example HCC827 or H2126) where we detected significant production of amphiregulin mRNA but not the protein by ELISA.

Fig. 2. High and low amphiregulin cell lines

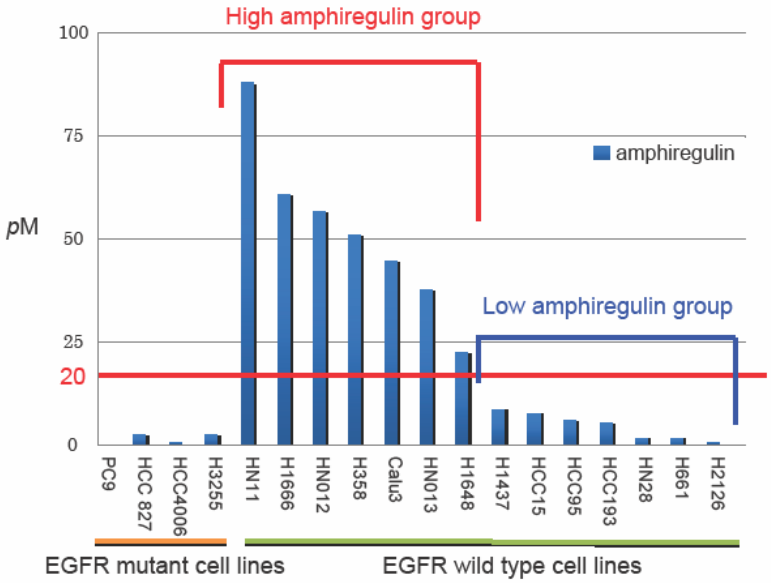
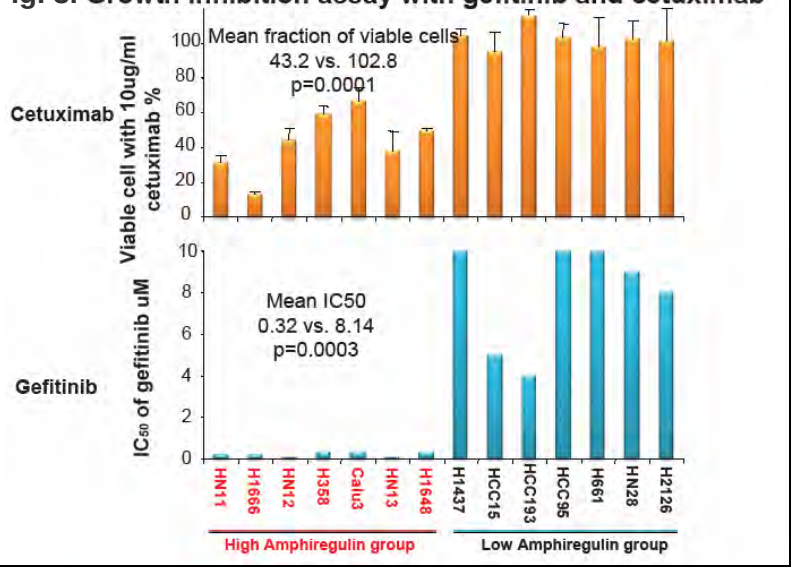


Fig. 3. Growth inhibition assay with gefitinib and cetuximab



We next studied the effects of gefitinib and cetuximab on the growth of 9 NSCLC and 5 HNSCC

cancer cell lines *in vitro* using the MTS assay (Figure 3). We compared the effects of these agents on cell growth in the high and low amphiregulin producing cells lines. As can be seen in Figure 3 in the top panel, 10 μg/ml of cetuximab inhibited the cell growth from 38-90%; only 4 of the cell lines were able to identify an IC_{50} value. Gefitinib was significantly more effective at inhibiting the growth of high amphiregulin producing cell lines compared to the low amphiregulin production as seen on the bottom panel (mean IC_{50} 0.32 μM vs. 8.14 μM, respectively; p = 0.0003; paired t-test). We thus chose to evaluate the effects of cetuximab on cell growth at 10 μg/ml, which is a concentration that is achievable in the plasma of cancer patients being treated with the standard cetuximab dosing regimen. As can be seen in Figure 3 in the upper panel, cetuximab more effectively inhibited the growth of high amphiregulin producing cell lines compared to those producing low amounts of amphiregulin (mean fraction of viable cells following cetuximab treatment (+/- standard deviation) 43.2 (+/- 18.1) vs. 102.8 (+/- 6.5), respectively; p = 0.0001; paired t-test).

Objective 3: Determine effects of TGF-α, EGF, and AR on the cell cycle and apoptosis of *EGFR* mutant and wild type cell lines.

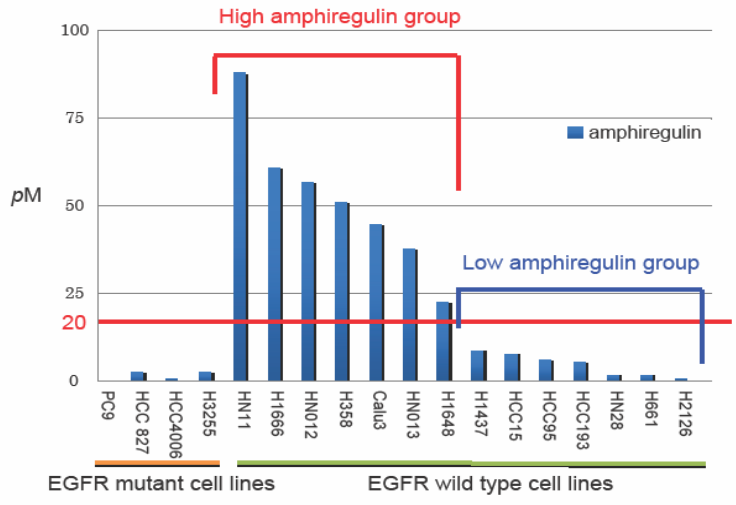
Methods:

Flow cytometry. We used fluorescence activated cell sorting (FACS) to analyze cell cycle distribution and to detect cell surface expression of amphiregulin. Briefly, 1-1.5 x 10^6 cells were seeded into 10 cm^3 plates, and indicated drugs were added 24 h later. The cells were then trypsinized and fixed overnight in ethanol at 4˚C. Fixed cells were then resuspended in 500 μg/ml RNase A (Sigma), centrifuged, resuspended in 69 μM propidium iodine (Sigma) in 38 mM sodium citrate, incubated at room temperature for 30 min, and analyzed by FACScan using Cell Quest software (Becton Dickinson Labware, Franklin Lakes, NJ). All experiments were repeated three times.

Cell surface detection of amphiregulin was performed. Cells were grown in 0.1% serum containing media for 24 hours, harvested and resuspended in PBS at 0.5 x 10^6 cells /mL. The anti-amphiregulin or control antibody (diluted in 3% BSA/PBS at 1:100 ratio) was added for 45 minutes, following which the cells were washed and exposed to an anti-goat secondary antibody at a 1:200 dilution for 45 minutes. The cells were washed in PBS and analyzed using FACScan as described above.

Results. The mechanism by which gefitinib and cetuximab treatment result in growth inhibition of high amphiregulin producing cell lines was studied using cell cycle analyses. Previous published experiments have shown that lung cancer cell lines with sensitizing mutations in the epidermal growth factor receptor (NCI-H3255) treated with gefitinib undergo apoptosis, as documented by an increased fraction in G_1 by cell cycle analysis and increased PARP cleavage by Western Blot analyses. For these studies, we used 1 μM gefitinib and

Fig. 4. High and low amphiregulin cell lines



10 μg/ml of cetuximab, both of which are achievable in the plasma of cancer patients at steady state who are being treated with these agents. In the high amphiregulin producing cell lines, both gefitinib (1 μM) and cetuximab (10 μg/ml) led to G1/S arrest without any evidence of apoptosis as assayed by FACS (Figure 4) or by Western blotting for cleaved PARP (data not shown). In contrast, in the low amphiregulin cell lines, cetuximab and gefitinib lead to either no significant increases or only minor changes in the G1/S phase of the cell cycle consistent with the lack of growth inhibition in this group of cell lines. In all of the cell lines in which there was an increase in the G1/S phase following cetuximab or gefitinib treatment, there was a corresponding decrease in the G2/M phase.

Objective 4: Determine effects of different *EGFR* mutations and EGFR inhibitors on phosphorylation of EGFR and downstream signaling intermediates.

Antibodies and Western Blotting. Cells were seeded at a density of 1 x 10^6 cells/ml and allowed to grow overnight in media containing 5-10 % FBS. The media was then replaced with RPMI containing 0.1 % FBS for 24 hours and then gefitinib or cetuximab were added to the media. The cells were incubated for another 2 hours, washed with PBS and lysed in buffer containing 25mM Tris (pH 8.3), 192mM glycine, 0.1% SDS and 1mM phenylmethylsulfonyl fluoride (PMSF). For studies evaluating the effects of gefitinib or cetuximab on phospho-EGFR, we used lysis buffer containing 1% SDS (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 1% SDS, 50 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride [PMSF], 25 μg/mL of leupetin, and 25 μg/mL of aprotinin] as described in our prior studies. After cell lysis, lysates were centrifuged at 16,000 *g* for 10 min at 4°C. The supernatant was used for subsequent procedures. Western blot analyses were conducted after separation by SDS/PAGE electrophoresis and transfer to nitrocellulose membranes. Immunoblotting was performed according to the antibody manufacturers' recommendations. Antibody binding was detected using an enhanced chemiluminescence system (New England Nuclear Life Science Products Inc.). Anti-phospho-Akt (Ser-473), anti-total Akt, and anti-EGFR antibodies were obtained from Cell Signaling Technology. The phospho-specific EGFR (pY1068), total ERK1/2 and phospho-ERK1/2 (pT185/pY187) antibodies were purchased from Biosource International Inc. The alpha-tubulin antibody was purchased from Sigma-Aldrich (St. Louis, MO).

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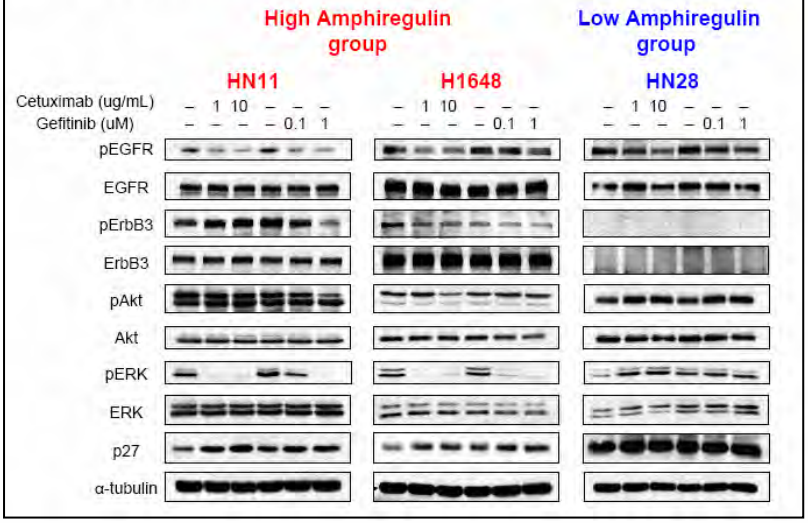
Results:

EGFR signaling was studied in the cancer cell lines with high and low amphiregulin at baseline and following treatment with cetuximab or gefitinib. The high amphiregulin producing cell lines expressed phosphorylated EGFR more frequently and to a greater degree than the low amphiregulin producing cells (Figure 5). In addition, Western blotting demonstrated the amphiregulin producing cells appeared to express greater amounts of total EGFR. Five

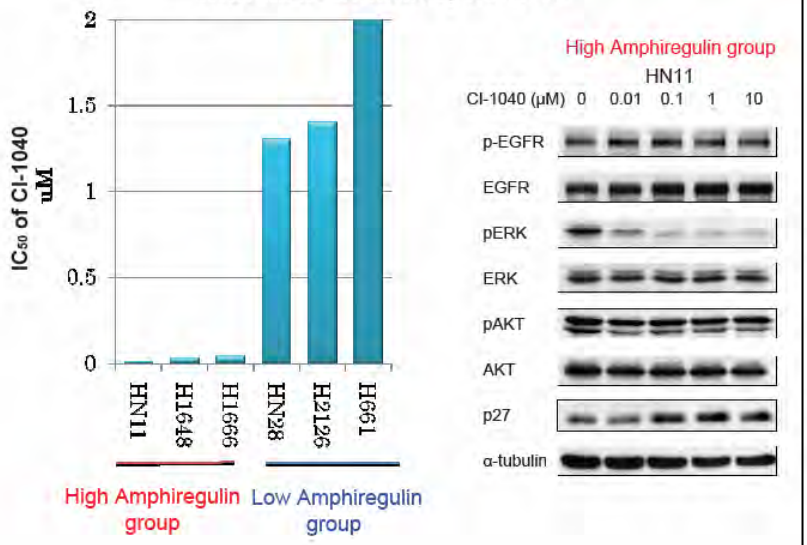
Fig. 5.
Effect to intracellular signaling with gefitinib or cetuximab



of the six high amphiregulin producing cell lines also expressed phosphorylated ERBB3 compared to only 1/6 of the low amphiregulin producing cell lines. Expression of phosphorylated ERBB3 has previously been shown to be associated with efficacy of gefitinib *in vitro* and all *EGFR* mutant gefitinib sensitive NSCLC cell lines express phosphorylated ERBB3. Furthermore, gefitinib treatment of *EGFR* mutant NSCLC cell lines leads to down regulation of ERBB3 and AKT phosphorylation.

The effects of gefitinib and cetuximab on EGFR signaling in 2 high amphiregulin producing cell lines (HN11 and H1648) and 1 low amphiregulin producing cell line (HN28) were examined. In both HN11 and H1648, gefitinib treatment led only to a minimal inhibition of ERBB3 and Akt phosphorylation (Figure 5). In contrast, gefitinib led to a more significant decrease in ERK 1/2 phosphorylation in these cell lines and an increase in p27 expression which is a known mediator of G1/S arrest. Similarly, cetuximab treatment did not result in inhibition of ERBB3 or Akt phosphorylation but did lead to downregulation of ERK 1/2 phosphorylation and an increase in p27 expression. Neither gefitinib nor cetuximab had a significant effect on AKT or ERK 1/2 phosphorylation in the H28 cell line (Figure 5). Together these findings suggest that gefitinib and cetuximab inhibit the growth of high amphiregulin producing cell lines by predominantly inhibiting ERK 1/2 signaling which leads to cell G1/S cell cycle arrest. We thus examined the effects of CI-1040, a specific MEK inhibitor, in the HN11, H1648 and H1666 cell line to determine if it could alone recapitulate the effects of gefitinib or cetuximab. All 3 cell lines were growth inhibited by CI-1040 (IC_{50s} 30-70 nM; data not shown). Treatment with CI-1040 led to a dose dependant inhibition of ERK 1/2 phosphorylation, an increase in p27 expression and to G1/S cell cycle arrest (Figure 6 and data not shown).

Fig. 6. Growth inhibition assay and immunoblotting with MEK inhibitor, CI-1040



Key Research Accomplishments:

- Thirty-five (35) patients participating in the BATTLE program have been allocated to the trial entitled, "A Phase II, Open Label Study of Erlotinib (Tarceva®) in Previously Treated Subjects with Advanced Non-Small Cell Lung Cancer."
- Amphiregulin is the agonist in tumor cell lines (both lung cancer and head and neck cancer cell lines) with wild type EGFR that is associated with response to gefitinib and cetuximab.

Reportable Outcomes:

Publications (Submitted)

- Yonesaka K, Zenullahu K, Lindeman N, Homes AJ, Jackman DM, Zhao F, Rogers AM, Johnson BE, Janne PA. Autocrine production of amphiregulin predicts sensitivity to both gefitinib and cetuximab in *EGFR* wild type cancers. Clinical Cancer Research, submitted.

Conclusions:

Amphiregulin is the agonist in tumor cell lines (both lung cancer and head and neck cancer cell lines) with wild type EGFR that is associated with response to gefitinib and cetuximab. These agonists and the determination whether the EGFR receptor is mutated will need to be studied in the tumor specimens from the patients participating in the phase II trial of erlotinib to see if these *in vitro* findings translate into the clinical specimens available from the patients participating in this study.

Specific Aim 2.2. Insulin-like Growth Factor Receptor Signaling Pathways and Resistance to Gefitinib in Non Small-Cell Lung Cancer Cells

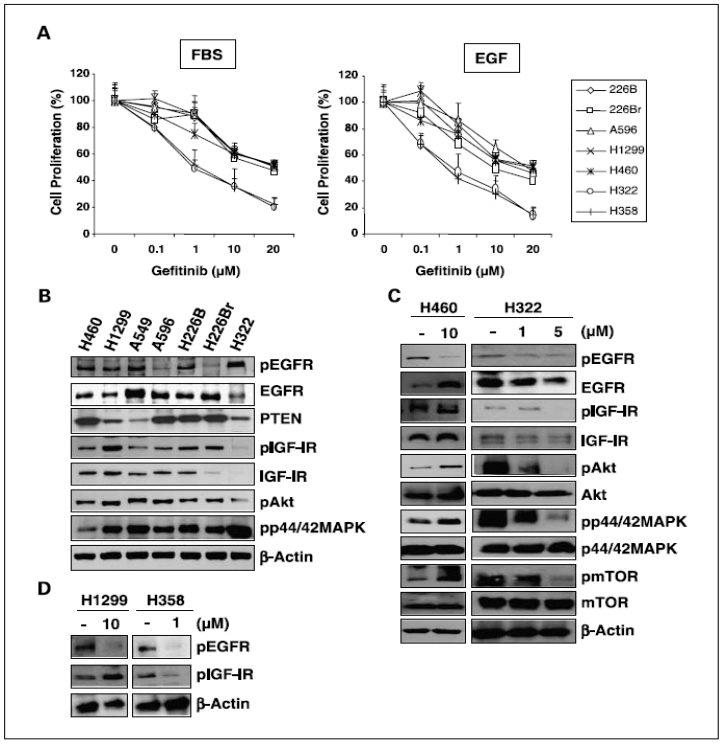
(PI: Ho-Young Lee, Ph.D.)

Non-small cell lung cancer (NSCLC) accounts for about 75%-80% of lung cancer cases and its dismal survival rate has not improved in the past 2 decades. The lack of effective therapy, the high proportion of patients with advanced disease at the time of diagnosis, and the rapidity of tumor progression are major contributors to lung cancer mortality, and raises the urgent need for novel strategies to treat this disease. Of many potential targets in adult solid tumors, the epidermal growth factor receptor (EGFR) has been extensively studied because overexpression of EGFR has been observed in a number of other common solid tumors including 40–80% of NSCLC (Jemal et al, 2003). Therefore, one therapeutic strategy was to use the agents targeting the EGFR pathway. However, negative results from several large-scale phase III clinical trials in lung cancer have been reported (Giaccone et al, 2002; Johnson, 2002), indicating the need for understanding the mechanisms that induce resistance to EGFR inhibitors. Accumulating evidence has implicated insulin-like growth factor receptor-I (IGF-IR) pathways in resistance to chemotherapy, radiation therapy, and molecularly targeted agents (Kulik et al, 1997; Lin et al, 1999; DiGiovanni et al, 2000; Porras et al, 1998; Toker and Newton, 2000). Our objective is to investigate whether IGF-IR and downstream signaling mediators, such as PI3K/Akt and MAPK, are involved in the resistance to anti-EGFR therapies in NSCLC.

<u>Update</u>

In the past year, we have investigated whether inhibition of the IGF-IR-mediated signaling pathway augments the antiproliferative effects of gefitinib on NSCLC cells *in vitro.* We determined the effects of gefitinib on cell proliferation in H596, H22B, H226Br, H460, H1299,

Figure 7. Stimulation of IGF-IR signaling pathway in gefitinib-resistant NSCLC cell lines. (A) MTT assay of indicated cell lines treated with gefitinib in media with 10% FBS or EGF for 3 d; repeated 3 times with representative shown. Points are mean of 8 wells; bars, upper 95% CIs. (B) Basal expression of (p)EGFR, (p)IGF-IR, (p)p44/42, (p)MAPK, (p)Akt and PTEN; (p) represents phosphorylation. Immunoblotting of downstream signaling components in H460 and H322 (C) and H1299 and H358 (D) cell lines treated with gefitinib. β–actin was a loading control.



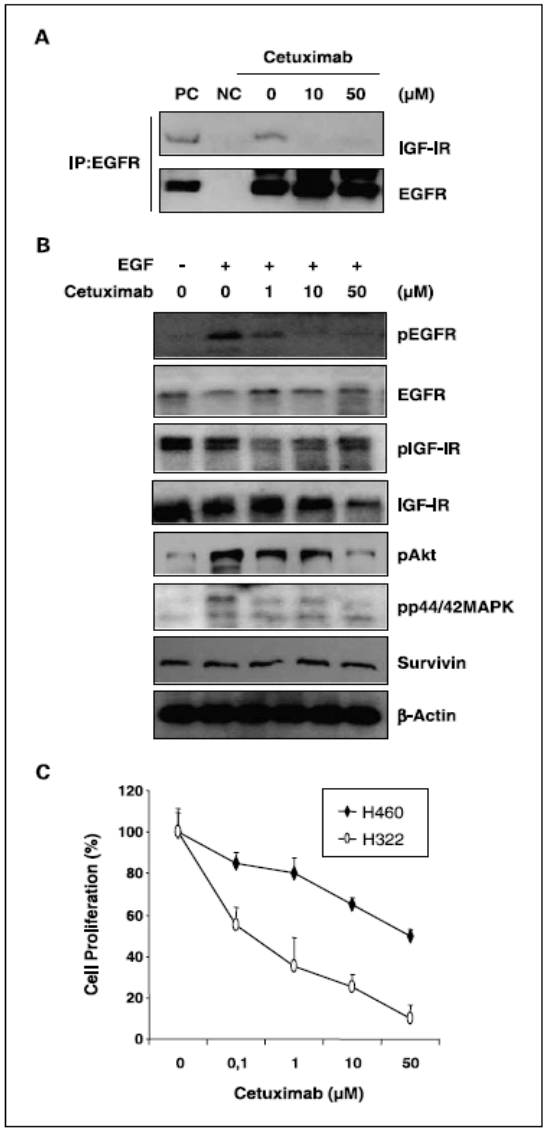


Figure 8. Effects of cetuximab on EGFR:IGF-1R interaction in NSCLC cells. (A) Effect of cetuximab on EGFR:IGF-IR heterodimerization and activation of the IGF-IR and EGFR signaling pathways. Whole-cell extracts (3 mg) from H460 cells were left untreated or treated with cetuximab (10 and 50 μM) for 3 d then immunoprecipitated with anti-EGFR antibodies and subjected to Western blot analysis with indicated antibodies. Contol precipitation was performed using mouse preimmune serum (PS) as a negative control. (B) Western blot analysis of survivin, pIGF-IR, IGF-IR, pEGFR, EGFR, pAkt, and pp44/4 MAPK expression in H460 cells treated with cetuximab (1, 2, 50 μM) for 3 d in absence or presence of EGF (50 ng/ml). (C) MTT assay of H460 and H322 cell lines after treatment with cetuximab in RPMI 1640 containing 10% FBS for 3 d. Points are mean value of eight identical wells of a representative single experiment; bars, upper 95% CIs.

H358, and H322 cell lines. The MTT assay revealed that gefitinib decreased NSCLC cell proliferation in a dose-dependent manner (Figure 7).

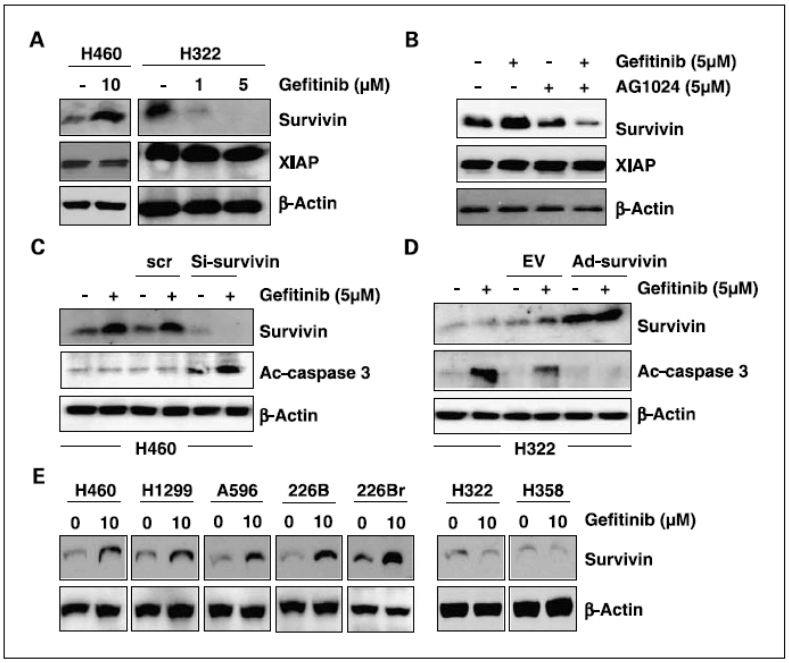


Figure 9. Role of survivin in the resistance of NSCLC cells to gefitinib. (A) Immunoblotting of survivin and XIAP in H460 and H322 cells treated with the indicated concentrations of gefitinib. (B) Effects of treatment with 5 mM gefitinib, 5 mM AG1024, or both on the expression of survivin and XIAP in H460 cells. (C, D) Effect of knockdown of expression or overexpression of survivin on H460 and H322 cells treated with gefitinib. H460 cells were transfected with scrambled (*scr*) or survivin siRNA and left untreated or treated with gefitinib for 48 h. H322 cells were infected with a control virus (Ad-Ev or Ad-survivin) and incubated for 3 d in the presence of gefitinib. Protein extracts were subjected to Western blotting for evaluation of survivin and active caspase-3. (E) Western blot analysis of survivin expression in NSCLC cell lines treated with 10 μM gefitinib. β-actin was a loading control.

The H322 and H358 cells were more sensitive to gefitinib than were the other cell lines. We next determined the mechanisms responsible for the sensitivity of NSCLC cells to gefitinib. We found that gefitinib inhibited NSCLC cell proliferation by inducing apoptosis when IGF-IR signaling was suppressed. Treatment with gefitinib, but not cetuximab, induced EGFR: IGF-IR interaction and activation of IGF-IR and its downstream signaling mediators, resulting in increased survivin expression in NSCLC cell lines with high levels of IGF-IR expression (Figure 8, previous page).

Inhibition of IGF-IR activation and knockdown of survivin expression led to increased apoptosis. In contrast, overexpression of survivin protected cells with low IGF-IR expression from gefitinib-induced apoptosis (Figure 9, next page). The two cells lines most sensitive to gefitinib treatment (H322 and H358) had the lowest levels of IGF-IR expression, suggesting that IGF-IR is involved in NSCLC cells' sensitivity to gefitinib. Most NSCLC tissues with EGFR overexpression had associated high levels of IGF-IR expression.

Key Research Accomplishments:

- Gefitinib inhibits HSCLC cell proliferation by inducing apoptosis when IGF-IR signalling was suppressed.
- Treatment with gefitinib, but not cetuximab, induced EGFR: IGF-IR interaction and activation of EGF-IR and its downstream signalling mediators, resulting in increased survivin expression in NSCLC cell lines with a high level of IGF-IR expression.
- Inhibition of IGF-IR activation and knockdown of survivin expression led to increased apoptosis.
- Overexpression of survivin protected cells with low IGF-IR expression from gefitinib-induced apoptosis. Most NSCLC tissues with EGFR overexpression had associated high levels of IGF-IR expression.

Reportable outcomes:

Publications

Morgillo F, Woo JK, Kim ES, Ciardiello F, Hong WK, **Lee H-Y**. Implication of the Insulin-like Growth Factor-1R Pathway in the Resistance of Non-small Cell Lung Cancer Cells to Treatment with Gefitinib. Clin Cancer Res, 13(9):2795-2803, 2007.

Conclusions:

Overall, our findings suggest that activation of IGF-IR and consequent induction of survivin expression contribute to the acquired resistance to EGFR tyrosine kinase inhibitors (TKIs), but not to the monoclonal antibody against EGFR in NSCLC cells. Suppression of IGF-IR signaling pathways may prevent or delay development of gefitinib resistance in patients with NSCLC.

Specific Aim 2.3. To investigate the molecular mechanisms of resistance to and biomarkers of the biologic activity of inhibitors of the VEGF pathway

(PI: John Heymach, M.D., Ph.D.)

The primary goals of this Aim were to develop biomarkers for the activity of VEGF inhibitors and investigate potential markers of therapeutic resistance. Substantial progress has been made towards these goals. The focus of our effort thus far has been in the development of our methodologies for the blood based biomarkers (objectives 2 and 3), in large part because of specimen availability. Notable advances over the past year, detailed below, include the following: 1) Development of techniques for assessing VEGF receptor-bearing monocytes by 7-color flow cytometry, and initial validation using clinical samples demonstrating that these populations are specifically modulated by VEGF inhibitors as compared to non-VEGF receptor bearing populations; 2) Development of methodologies for assessing different circulating endothelial cell populations, and 3) Application of plasma profiling of angiogenic factors to identify potential mechanisms of resistance to VEGF inhibitors. In particular, we have developed a profile of cytokine and angiogenic factors and, in our initial application of this platform, we have identified the HGF/MET axis as potentially being involved in resistance to ZD6474.

The objectives of this aim have not been modified since the project began. Progress on these objectives is detailed below.

Objective 1: Quantitatively assess VEGFR phosphorylation, downstream signaling, and biomarkers of angiogenesis in pre- and post-treatment tumor biopsy samples.

<u>Update</u>

This objective requires quantitative analysis of tumor specimens pre- and post-treatment from patients enrolled in the BATTLE protocol using laser scanning cytometry to detect angiogenesis, endothelial apoptosis, and VEGF receptor phosphorylation. Our focus this year was in refining our methods for this analysis and applying them in preclinical models. Using human xenograft tumors (H1975 and A549) in mice, we have now developed tumors with resistance to VEGFR inhibitors. Our preliminary results suggest that one possible mechanism of resistance is “endothelial switching” in which endothelial cells change from being VEGF-dependent to EGFR- or PDGFR-dependent. This phenomenon has been observed in tumors that have become

resistant to ZD6474 or bevacizumab, and is consistent with a study we recently published in melanoma in collaboration with Dr. Michael Klagsbrun. Based on this analysis, our upcoming analysis of BATTLE specimens will include not only changes in VEGFR activation, but analyses to determine whether the same mechanism of endothelial switching contributes to resistance. We anticipate the analysis of the available clinical tumor specimens from patients treated in the BATTLE protocols will be done in a series of batch analyses in the upcoming year.

Objective 2: Investigate the utility of circulating endothelial cells (CECs), monocytes, and other cells in peripheral blood as biomarkers for antiangiogenic activity and inhibition of the VEGF pathway.

Update

A major focus of our efforts has been to develop methods for identifying specific populations of circulating endothelial cells (CECs), circulating endothelial progenitors (CEPs), and monocytes, particularly those bearing VEGF receptor 1 (VEGFR1+ monocytes), which may serve as potential biomarkers for VEGF inhibitors. Previously, using our earlier 4-color flow cytometry method, we established that in patients treated with the VEGFR inhibitor sunitinib, both CECs and monocytes were modulated during treatment. These changes differed in patients who had progressive disease and clinical benefit, suggesting that these may be useful biomarkers for VEGFR inhibitors (Norden-Zfoni et al., 2007; Desai et al. 2007).

This year, while awaiting further samples to be collected on the trials, we further improved out analysis by refining an 8-color flow cytometry method for measuring CECs and VEGFR1+ monocytes as well as other monocyte populations bearing targeted receptors. Results from analysis of our methods to specimens from clinical trials of VEGFR inhibitor AZD2171 demonstrated that these populations are specifically targeted in patients treated with AZD2171 (Wu et al, Proc ASCO 2008) and by sunitinib (Zurita et al, 2007). These results were presented at AACR and ASCO meetings this past year.

Given that we have now validated the potential utility of these new markers in other studies of VEGFR inhibitiors, we will now apply these markers to assess samples that are currently being collected and batched from the BATTLE protocol.

Objective 3: Systematically examine changes in the plasma and serum angiogenic profiles consisting of a panel of proangiogenic cytokines, targeted receptors, and potential biomarkers of endothelial damage.

Update

In the first phase of the BATTLE clinical trial, 72 patients with advanced, previously treated NSCLC were enrolled into one of four trials based on their pre-treatment tumor biomarker profile. The four biomarker groups were EGFR (mutation, gene amplification, pEGFR and ErbB3), angiogenesis (VEGF pathway, VEGF and VEGFR-2), K-ras/B-raf mutations, and RXR/Cyclin D-1. We performed exploratory analysis of plasma levels of 59 cytokine/angiogenic factors (CAFs) to investigate potential correlation with pathway activation from pretreatment tumor specimens. Sixty-two (62) of the 72 patients consented to the optional blood collection for CAFs analysis.

We used multiplex bead assays (Biorad, Hercules, CA) to measure 50 plasma CAFs, including VEGF, bFGF, HGF, E-selectin, MMP-9, multiple chemokines and interleukins (ILs). Multiplex bead-based technology enables the simultaneous quantitation of up to 100 analytes. This

technology uses polystyrene beads internally labeled with differing ratios of two spectrally distinct fluorophores. Each fluorophore can have any of 10 possible levels of fluorescent intensity; thereby creating a family of 100 spectrally addressed bead sets. These assays contain labeled beads conjugated with monoclonal antibodies specific for a target protein or peptide such as a cytokine or a phosphoprotein. Each of the 100 spectrally addressed bead sets can contain a capture antibody specific for a unique target protein. The antibody-conjugated beads are allowed to react with sample and a secondary, or detection, antibody in a microplate well to form a capture sandwich immunoassay. Multiplex assays can be created by mixing bead sets with different conjugated antibodies to simultaneously test for many analytes in a single sample. The use of this technique has been well documented in the literature and results are comparable to that of ELISA [1-3]. Analysis of these factors have been completed for up to 23/27 proteins (Bio-Rad) from a single specimen and from various matrices including: human serum and plasma and cell media from human cancer cell lines using Bio-Plex 200 system (Bio-Rad Laboratories, Hercules, CA) with the Bio-Plex Manager software. Currently up to 50 human cytokines can be analyzed from 2 separate kits (23-plex & 27-plex). Depending on which target protein, a typical calibration curve is generated covering the dynamic range from 2 to 32,000 pg/mL. Typical sample volume required for each sample well ranges from 50 – 100 μL. For each plate, the standard curve is assessed to ensure that the expected assay range was achieved. For each individual sample, the mean concentration is calculated for duplicate samples, and the coefficient of variance % (CV%) calculated for each of the analytes. If the median CV% is greater than 25%, analysis of the sample was repeated. In the rare case that the repeat CV% is greater than 25%, one of the two analyses will be selected based on lower CV% and consistency with prior values.

Table 2. Cytokines and Chemokines assayed using multiplex bead assay.

Human Cytokines/Chemokines Profiling			
pro/antiangiogenic factors	**EGF axis**	**chemokines**	**Interleukins**
VEGF	EGF	MCP-1 (MCAF)	IL-1α
FGF-basic	TGF-α**	MCP-3	IL-1β
HGF		RANTES (CCL5)	IL-1RA
EGF	**IGF axis**	MIP-1α	IL-2
PDGF-BB	IGF-I**	MIP-1β	IL-2Rα
MMP-9*	IGF-II**	MIP-2	IL-3
	IGF-BP3**	MIG (CXCL-9)	IL-4
endothelial function/damage		Eotaxin (CCL11)	IL-5
sVEGFR-2**	**hypoxia**	IP-10 (CXCL10)	IL-6
sE-selectin	Osteopontin*	SDF-1α (CXCR4)	IL-7
VCAM-1	HIF-1**	KC (CXCL1)	IL-8
		GRO-α	IL-9
inflammation/adhesion	**growth factors**	CTACK (CCL27)	IL-10
ICAM-1	GM-CSF		IL-12 (p40)
MIF	G-CSF		IL-12 (p70)
TNF-α	M-CSF		IL-13
TNF-β	SCGF-β		IL-15
IFN-α	SCF		IL-16
IFN-γ	Beta-NGF		IL-17
LIF			IL-18

*Conducted using Cytokine 23-Plex & 27-Plex (Bio-Rad) unless otherwise stated. * by 3-plex from Lincoplex, ** by standard ELISA assay (R&D, Invitrogen and DSL)*

Human cytokines profiling a conducted using the following methods: Analysis by the use of bead-based multiplex will utilize cytokine 27-Plex (Bio-Rad catalog #171A11127) & 23-Plex (Bio-Rad catalog #171A11123) and Human CVD Biomarker Panel 1 (3-plex includes MMP-9, sICAM-1, and sE-Selectin) from LincoPlex (Catalog # HCVD1-67AK). The remainder of analytes are determined using by validated, enzyme-linked immunosorbent assays (ELISA) assays. Soluble VEGFR1 and sVGFR2 are analyzed by EIA (Invitrogen, Carlsbad, CA). Total IGF-I, IGF-II and IGFBP-3 is measured by ELISA assays with an extraction method (DSL, Webster, TX, USA). Free IGF-I (unbound, DSL) is determined by using a free IGF-I ELISA immunoassay with an antibody that binds specifically to free-IGF-I from plasma samples. Human Osteopontin (OPN) is analyzed using the Quantikine ELISA Kit (R & D Systems). The factors profiled are summarized in the table above (Table 2).

To evaluate the association between the CAFs and pretreatment tumor markers, Wilcoxon test was performed. A significantly higher score of association was observed with osteopontin (p=0.005), IL-2Rα (p=0.012), IL-6 (p= 0.026) and IL-7 (p=0.031) in patients in the EGFR positive biomarker group, compared to those patients who were negative for this marker group (Figure 10). Higher IGFBP-3 was significantly associated with K-ras/B-raf mutations (p=0.007) (Figure 11) and IL-12 (p=0.021) was significantly higher in the angiogenesis positive marker group (Figure 12). Additionally, MMP-9 (p=0.017) was significantly higher in patients who were negative for angiogenesis markers. Additional analyses will be completed once an additional cohort of samples have been collected and at the completion of enrollment to see whether these same markers continue to correlate within each tissue-based biomarker group. At completion of the study, we will complete two other analyses: 1) evaluate the modulation of CAFs by each treatment arm to identify activity-based (treatment-based) profiles, and 2) evaluate for potential correlation between outcome measures such as progression-free survival (PFS) with CAFs to look for potential predictive signatures for each treatment group.

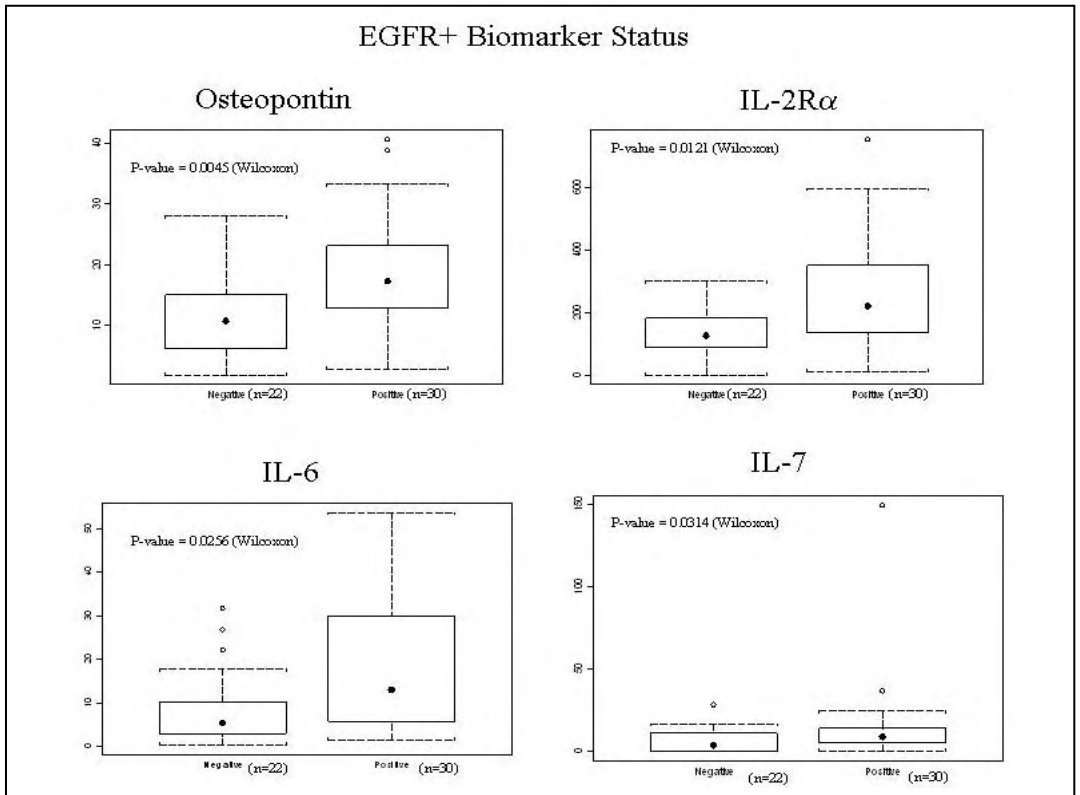


Figure 10. CAF Profile for Patients who were positive for EGFR biomarkers. Correlation between EGFR positive status from pretreatment tissue analysis and concentration of four protein factors (osteopontin, IL-2Rα, IL-6, IL-7) from pretreatment plasma samples.

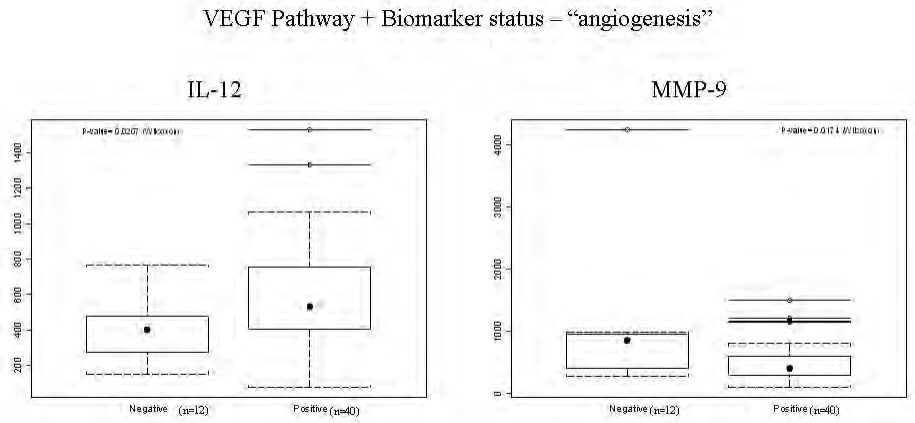


Figure 11. CAF Profile for Patients who were positive for VEGF pathway biomarkers. Correlation between VEGF pathway activation status from pretreatment tissue analysis and concentration of IL-12 and MMP-9 pretreatment plasma samples.

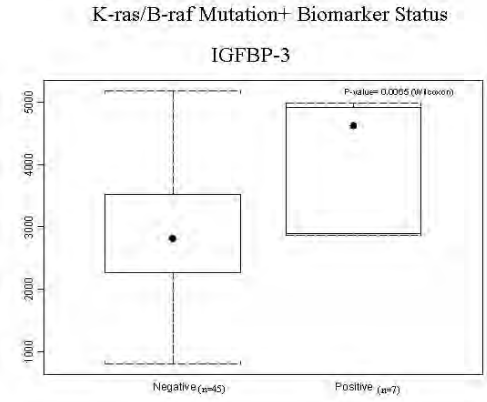


Figure 12. CAF Profile for Patients who were positive for K-ras/B-raf mutations. K-ras/B-raf mutation status: Correlation between K-ras/B-raf mutational status from pretreatment tissue analysis and concentration of IGFBP-3 pretreatment plasma samples.

Because sorafenib and ZD6474 are two agents being tested in the BATTLE study, we investigated whether we could develop signatures in other studies with these agents and then validate them in BATTLE. In one study, we investigated whether CAF profiling could identify patients likely to receive greater benefit from ZD6474 (vandetanib) in combination with chemotherapy from a randomized phase II study (Heymach et al, 2007). Although no one single marker was highly predictive of benefit, a combination signature of 5 markers was predictive. Using this signature, patients with a score of less than 2 had an HR of 0.35 (compared to 0.76 for the overall population), while those with a score $\geq$ 2 had a significantly higher HR (HR 1.11; p value 0.13 for interaction) (Figure 13; Hanrahan et al, manuscript in preparation). Using a similar approach, we derived CAF markers of benefit from a trial of sorafenib in renal cell carcinoma (Zurita et al, 2008). We will test the same approach, and see whether markers from this trial can be validate, using the BATTLE specimens as soon as the clinical outcome data becomes available.

Key Research Accomplishments:

- Successfully collected between 77% to 100% (91% overall) of potential samples (consent is optional) at various time-points during course of treatment.

- Measured and analyzed the first group of baseline plasma samples using multiplex bead-based and ELISA assays
- Preliminary data to be presented as an oral presentation in the Clinical Science Symposium "Proteomic Investigations in Lung Cancer" at the upcoming 2008 ASCO Annual Meeting

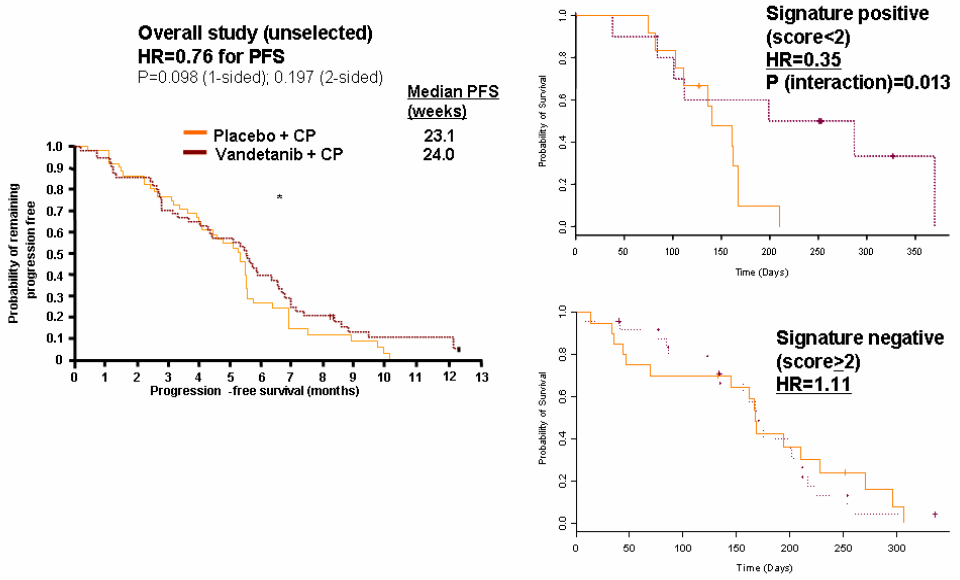


Figure 13. CAF signature identify groups that have different PFS benefit from ZD6474 (vandetanib) in combination with chemotherapy compared to chemotherapy alone. Note that in the overall study population a hazard ratio of 0.76 was observed for the ZD6474 arm compared to the control arm. No single marker was highly predictive but, using a signature consisting of the top five CAF markers, the benefit is significantly larger in the signature positive group with a HR of 0.35 compared to 1.11 in the signature negative group (p value for interaction 0.013) . Heymach et al, Proc IASLC, 2007; Hanrahan et al, manuscript in preparation.

Reportable Outcomes:

In collaboration with PIs of Clinical Trials in Aim 1, Biomarker and Biostatistics Core, and Li Mao, Aim 3:

Abstracts

Tran HT, Kim ES, Lee JJ, Herbst RS, Liu S, Wistuba II, Yan S, Mao L, Hong WK, Heymach JV. Correlation between plasma cytokine/angiogenic factors (C/AF) and signaling pathways activation from baseline tumor biopsy specimens in patients with advanced non small cell lung cancer (NSCLC): preliminary analysis from the Biomarker-based Approaches of Targeted Therapy for Lung Cancer Elimination (BATTLE) clinical study. Abstract 8010, Proc ASCO 2008.

Conclusions:

Several plasma CAFs are associated with specific tumor-derived pathway activation. This preliminary study suggests that broad-based plasma profiling of cytokines and angiogenic

factors may be a feasible approach for identifying markers of activation of tumor signaling pathways. In addition to the evaluation of pathway activation using plasma samples, we will be evaluating modulation of CAFs by each treatment arm, evaluating for potential predictive plasma signature(s) with clinical outcome measures such as progression-free survival (PFS). The final step will be to validate the plasma predictive signature derived from BATTLE with other randomized clinical studies.

Specific Aim 2.4. To investigate the molecular mechanisms of the effects of the combination of bexarotene and erlotinib on NSCLC cells

(PI: Reuben Lotan, Ph.D.)

The need to discover and introduce more effective treatment agents and combinations is urgent, as is the need to improve the selection of the right agent or combination of agents for each patient on the basis of our understanding of the molecular targets. The combination of the retinoid X receptor (RXR)-selective ligand Bexarotene and the epidermal growth factor receptor (EGFR) tyrosine kinase (TK) inhibitor erlotinib appears to be a promising approach, and it will be tested in patients with NSCLC in the BATTLE program. Some aspects of the mechanisms of action of these two agents are not fully resolved. Therefore, we propose to investigate how they exert their effects on NSCLC cells so as to improve their usefulness in future clinical trials.

The objectives of this project have not changed.

Objective 1: To determine by immunohistochemical analysis the expression of nuclear receptors (retinoic acid receptors [RAR]-α, -β, and -γ; RXR-α, -β, and -γ; and PPAR-γ1 and PPAR-γ2) and cyclin D1 in NSCLC specimens obtained from patients to be enrolled in the BATTLE umbrella trial and from patients whose cancer progresses on treatment.

Update

During the last year, we continued to collaborate with Dr. Ignacio Wistuba (Director of the Biomarker Core) and completed the analysis of samples of NSCLC from 112 patients. The patients included 75 (67%) with a history of smoking tobacco. Paired adjacent normal epithelium was also available for a few of these patients. All lesions were reviewed histopathologically by Dr. Wistuba to confirm the original diagnosis. The avidin-biotin-peroxidase complex method was used to detect immunoreactivity for retinoid receptors (RXRs) using affinity-purified rabbit polyclonal antibodies against RXR-α, -β, and -γ of human origin (Santa Cruz Biotechnologies, Santa Cruz, CA, USA). Dr. Wistuba and a second histopathologist examined independently all the stained histological sections to establish the nuclear and cytoplasmic staining patterns for each RXR. The expression was scored by multiplying the intensity (range 1 to 3) and the proportion of stained tumor cells among the total cell population in several microscopic fields. A sample was considered to have as a high expression level if given a score greater than 2. A statistically significant higher median percent ($p=0.001$) and lower median RXRγ cytoplasmic intensity ($p<0.001$) were observed in the squamous cell carcinoma as compared to adenocarcinoma (data not shown). A trend was observed for the loss of nuclear RXRα protein across the different pathological stages of NSCLC; higher TN stage had lower level of nuclear RXRα ($p=0.04$) (Figure 14). Furthermore, a significant decrease in the cytoplasmic staining of RXRβ

Figure 14. Association between Nuclear expression of RXRα and PatientsÕTumor Stage

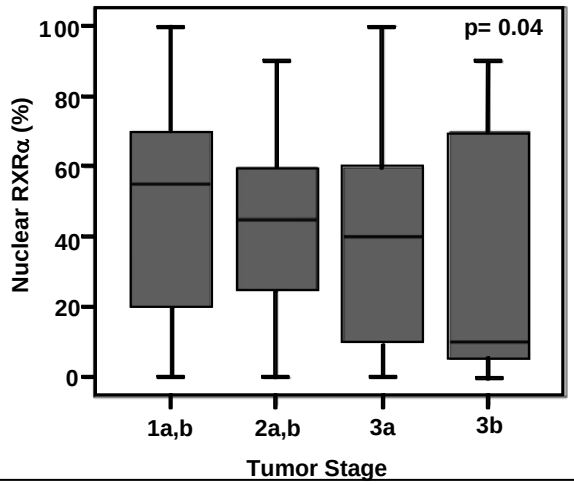
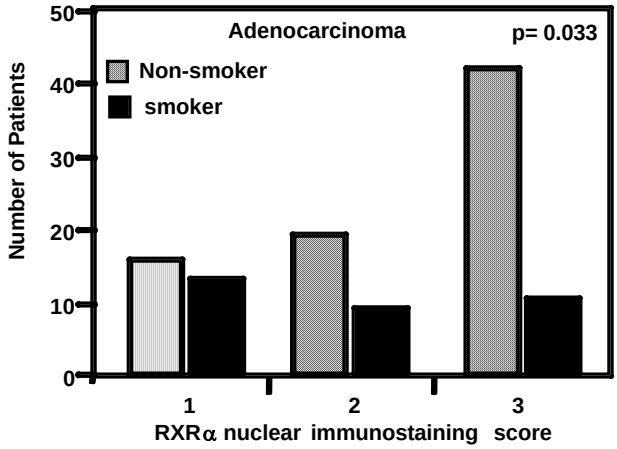
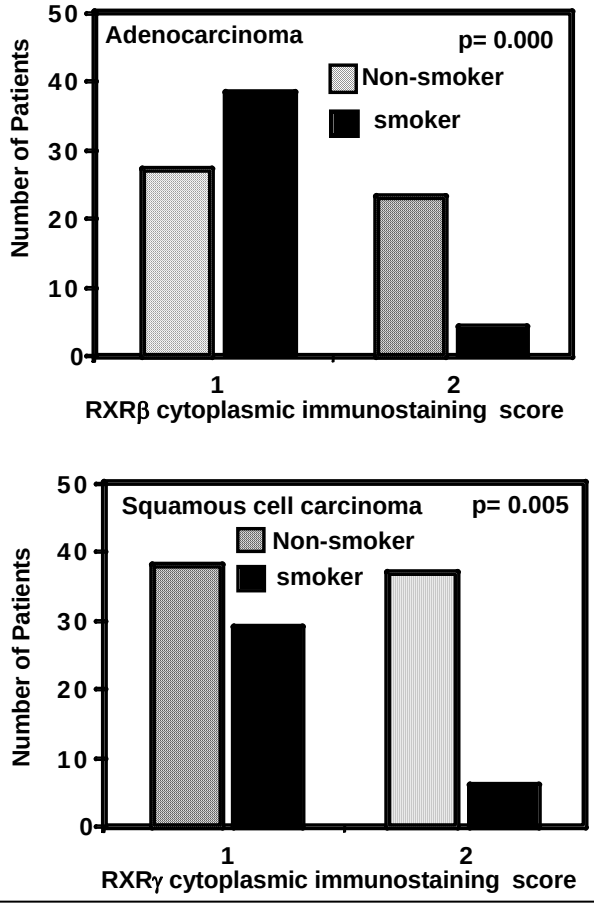


Figure 15. Associations between nuclear expression of RXRα and smoking history of patients with adenocarcinoma.



(p=0.031) and RXRγ (p=0.024) was observed in stage III tumors as compared to stage I and stage II tumors, respectively (data not shown). Because smoking is a major cause of lung cancer, we analyzed the data with respect to smoking status and found a significant loss in the nuclear expression of RXRα (p=0.033) and cytoplasmic staining of RXRβ (p<0.001) proteins in adenocarcinoma patients having history of smoking. On the other hand, a decrease in cytoplasmic RXRγ immunostaining was observed in squamous cell lung carcinoma patients who are smokers (p=0.005) (Figures 15 and 16). Our results show that there is an anomalous expression of RXR receptor subtypes in NSCLC and this anomaly correlates with tobacco exposure. Altered retinoid receptors expression may play a role in the pathogenesis and progression of these tumors because various retinoid-controlled pathways, including cellular differentiation and cell cycle control depend on intact receptor function. Similarly, decreased level of RXRs in smokers may compromise their response to the RXR ligand Bexarotene in the clinical trial.

Figure 16. Association between Cytoplasmic expression of RXRβ and RXRγ and Smoking History of lung cancer Patients

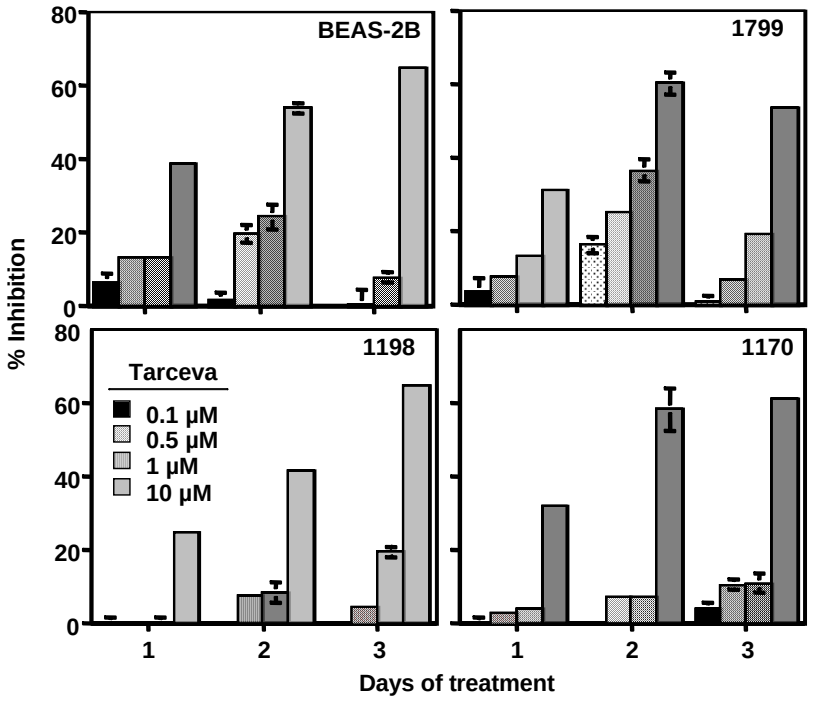


Objective 2: Examine the effects of bexarotene, erlotinib, and rosiglitazone alone and in combination on the growth and apoptosis of NSCLC cells, cyclin D1 and PPAR-γ levels, and gene expression profiles.

Update

Effects of the Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor Tarceva (erlotinib) on human premalignant and malignant lung cells. We examined the effects of Tarceva on cells representing two *in vitro* models for lung carcinogenesis. The first model consists of cells immortalized using a viral SV40 large T antigen/adeno12 (BEAS-2B and 1799), and further transformed (1198) by exposure to cigarette smoke condensate (CSC) *in vivo*. Tumorigenic (1170-I) cells were also derived from the BEAS-2B exposed to CSC *in vivo* (Reddel et al., 1988; Klein-Szanto, 1197). In this model, the 1198 and 1170-I cells express higher levels of the EGFR than the BEAS2B and 1799 cells. Nonetheless, all cell lines showed similar sensitivity to the highest dose of Tarceva (10 μM) and the BEAS-2B and 1799 cells were even more sensitive than the 1198 and 1170-I cells at the lower dose of Tarceva (1 μM) (Figure 17). The second model consists of cells derived from normal human bronchial epithelial cells by immortalization by overexpression of Cdk4 and hTERT and subsequent transfection of oncogenic K-RAS, or mutant EGFR and knockdown of p53 by shRNA (Sato et al., 2006). Such cells show progressive transformed properties but are not tumrigenic even when multiple genetic changes are inflicted on them. Dr. John Minna's group, with whom we collaborate on this project, has already found that these cells are exquisitely sensitive to EGFR inhibition. We confirmed their finding in our laboratory (data not shown) and, in the future, we will use them for treatment with combinations of Tarceva and Bexarotene a PPARγ ligands.

Figure 17. Time- and dose-dependent inhibition of the growth of immortalized, transformed and tumorigenic lung epithelial cells by Tarceva (erlotinib)



Objective 3: Determine whether RXRs, EGFR, and PPAR-γ are required to mediate the effects of bexarotene, erlotinib, and rosiglitazone, respectively, on cell growth control and apoptosis, and examine the functional significance of changes in gene expression induced by receptor agonists used singly or in combinations.

Update

Effects of Bexarotene on gene expression in lung cancer cells. A recent report described that Bexarotene (1 μM) increased the expression of several genes, including the tumor suppressor RAI3 (also known as GPRC5A), in normal human mammary epithelial cells (HMEC) (Li Y et al., 2008). We are very interested in the GPRC5A gene encoding a seven transmembrane protein, possibly a G protein coupled receptor, because our laboratory was the first to identify and clone this gene in 1998 (Cheng and Lotan, 1998) and we demonstrated that GPRC5A is expressed preferentially in normal human lung tissue and its expression is decreased in a variety of human lung cancers (Tao et al., 2007). More importantly, we have generated knockout mice in which both alleles of the Gprc5a gene have been deleted and found that such mice develop lung tumors spontaneously indicating that Gprc5a acts as a tumor suppressor. Therefore, we wanted to determine whether Bexarotene can induce GPRC5A in human lung cells and, indeed, we found using RT-PCR that Bexarotene can increase the mRNA levels of Gprc5a in human NSCLC cell line 1792 (Figure 18). We have also analyzed 11 NSCLC cell lines using western blotting to determine the levels of GPRC5A, which is expressed as an unglycosylated 40kDa protein and has several higher molecular weight bands, it's glycosylated forms, and the levels of EGFR and its related receptor HER2. Actin was used as a loading control (Figure 19). We found that 4 of the 11 cell lines had low or no GPRC5A, whereas, the remaining 7 cell lines expressed constitutively different levels and molecular forms of GPRC5A (Figure 19). Interestingly, it appears that those cell lines that expressed higher levels of GPRC5A also expressed higher levels of EGFR. Only three cell lines expressed high levels of HER2. It has been reported recently that GPRC5A in normal human mammary epithelial cells treated with epidermal growth factor can be phosphorylated on tyrosine residue in the cytoplasmic tail region making GPRC5A a substrate for EGFR family members EGFR and HER2 (Zhang Y et al., 2005; Wolf-Yadlin A et al., 2006). To determine whether this is also the case in lung cells, we treated human NSCLC cell line H292, which express GPRC5A constitutively as seen in Figure 19, with EGF and found that GPRC5A is indeed phosphorylated (data not shown). This suggests that there may be cross-talk between EGFR signaling and GPRC5A signaling and that Bexarotene may affect this cross-talk by increasing the expression of GPRC5A in cells where it is suppressed. We will pursue this mechanism in the coming year as it may be relevant to the effects of Bexarotene in the patients.

Figure 18. Dose- and time-dependent induction by bexarotene of GPRC5A mRNA in 1792 NSCLC cells (RT-PCR).

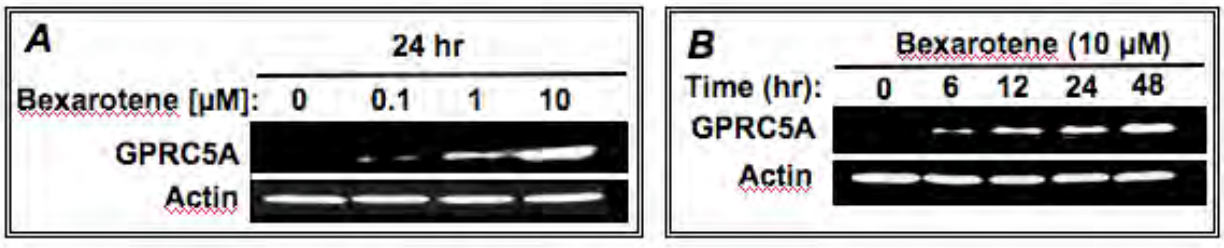
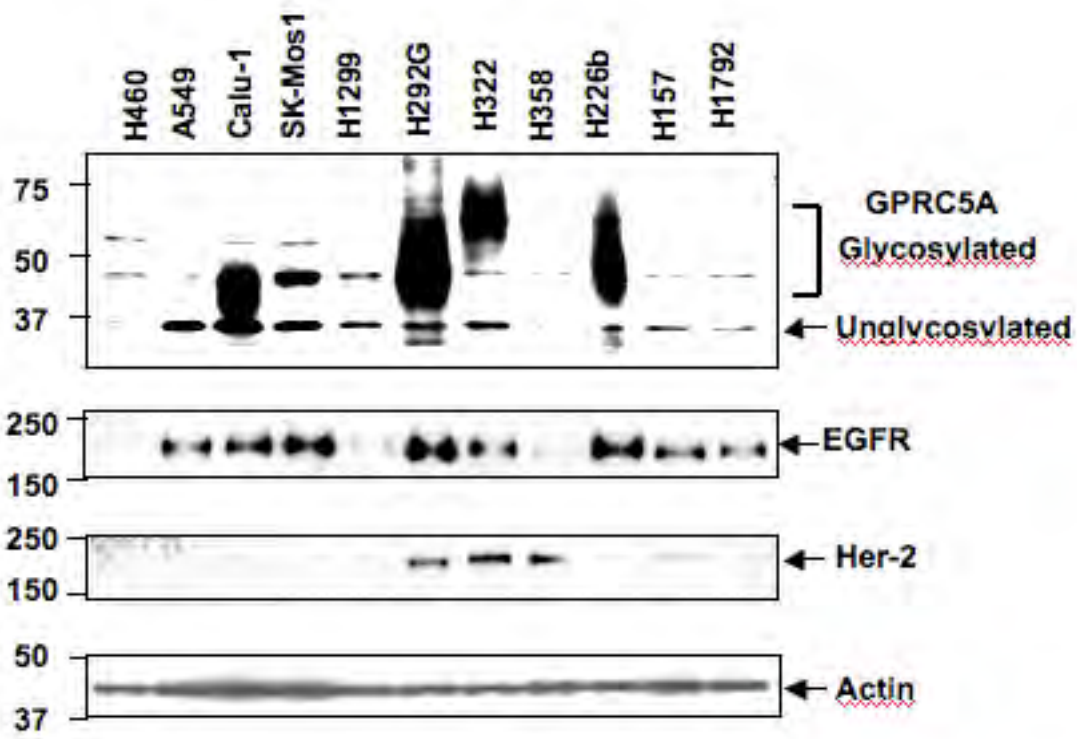


Figure 19. Analysis of the expression of GPRC5A, EGFR, HER2, and actin in 11 NSCLC cell lines by western blotting.



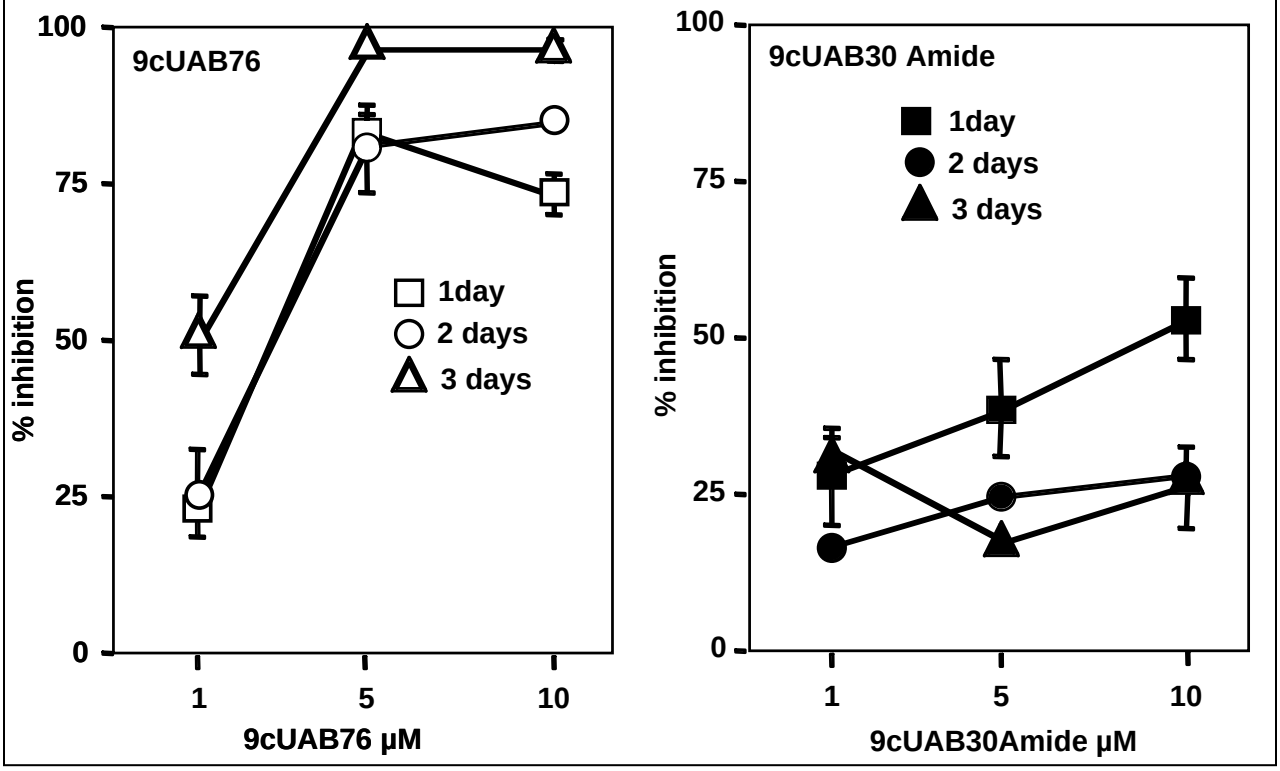
Objective 4: Evaluate the growth inhibitory effects and mechanisms of action of novel RXR ligands AGN194204 and 9cUAB30 alone or combined with erlotinib and rosiglitazone on NSCLC cells.

Update

Investigation of the effects of retinoids and rexinoids on lung cells. Because Bexarotene is not a pure RXR agonist and at high doses can also bind and activate RARs, we decided to examine the effects of RXR ligands that only bind and activate RXRs and have reduced triglyceride elevating effects compared to Bexarotene. To this end, we have established a collaboration with Dr. Donald Muccio (Department of Chemistry, University of Alabama) who has synthesized several novel conformationally constrained rexinoids [e.g.,(3',4'-dihydro-1'(2'H)-naphthalen-1'-ylidene)-3,7-dimethyl-2,4,6-octatrienoic acid (9cUAB30); Atigadda, V.R. et al., 2003] and has demonstrated that they are more potent than Bexarotene in chemoprevention trials in animal models and do not increase triglyceride as much as Bexarotene does. We received six retinoids including a novel RXR agonist 9cUAB76 and a RARβ agonist 9cUAB30Amide. We tested the effects of these retinoids on the growth of immortalized human lung cells BEAS2B and found that while the RXR agonist was effective even at 1 μM dose giving 50% inhibition after 3 days of treatment and nearly 100% inhibition after 3 days of treatment with 5 μM (*in vivo* blood levels of 8 μM have been obtained 3 hr after oral dose of this retinoid to mice; D. Muccio, personal communication), the RARβ agonist did not cause more than 50% inhibition after 3 days of treatment with even 10 μM dose (Figure 20). The marked growth inhibition observed already after 1 day of treatment with the RXR agonist suggests that this rexinoid induces cell death. We will test this in the coming year. Our results indicate that the

RXR agonist may be superior to RAR selective agonists against lung cancer. We will continue to explore the activity of the 9cUAB76 against a collection of NSCLC cell lines available to us.

Figure 20. Dose and time-dependent growth inhibitory effects of the synthetic retinoids 9cUAB76 (RXR agonist) and 9cUAB30 Amide (RARβ agonist) on the growth of immortalized human lung cells (BEAS-22B).



Key Research Accomplishments:

- The level of the nuclear RXRα and the cytoplasmic RXRβ and RXRγ protein decreases across the different pathological stages of NSCLC; higher TN stage had lower level of RXRs.
- The level of the nuclear RXRα and the cytoplasmic RXRβ (in adenocarcinomas) and RXRγ protein (in SCCs) decreases with tobacco exposure.
- The sensitivity to growth inhibition by Tarceva of more progressed cells (transformed and tumorigenic) with elevated expression of EGFR was not higher than that of immortalized cell lines.
- Bexarotene can induce the tumor suppressor GPRC5A in human lung cancer cells.
- Cell lines that expressed higher levels of GPRC5A also expressed higher levels of EGFR and GPRC5A is phosphorylated in cells treated with EGF.
- The novel RXR agonist 9cUAB76 is much more potent in growth inhibition of premalignant lung cells than a RARβ agonist (9cUAB30Amide). The marked growth inhibition observed already after 1 day of treatment with the RXR agonist suggests that this rexinoid induces cell death.

Reportable Outcomes:

None

Conclusions:

Altered retinoid receptors expression may play a role in the pathogenesis and progression of these tumors because various retinoid-controlled pathways, including cellular differentiation and cell cycle control depend on intact receptor function. Similarly, decreased level of RXRs in smokers may compromise their response to the RXR ligand Bexarotene in the clinical trial.

This suggests that there may be cross-talk between EGFR signaling and GPRC5A signaling and that Bexarotene may affect this cross-talk by increasing the expression of GPRC5A in cells where it is suppressed. We will pursue this mechanism in the coming year as it may be relevant to the effects of Bexarotene in the patients.

Our results indicate that the RXR agonist may be superior to RAR selective agonists against lung cancer. We will continue to explore the activity of the 9cUAB76 against a collection of NSCLC cell lines available to us.

Specific Aim 3: To identify biomarkers as novel predictors of clinical end points and potential therapeutic targets

(PI: Li Mao, M.D.)

Objective 1: Identify molecular features in tumor tissues that correlate with patients' responses to individual regimens used in the clinical trials of the proposed program.

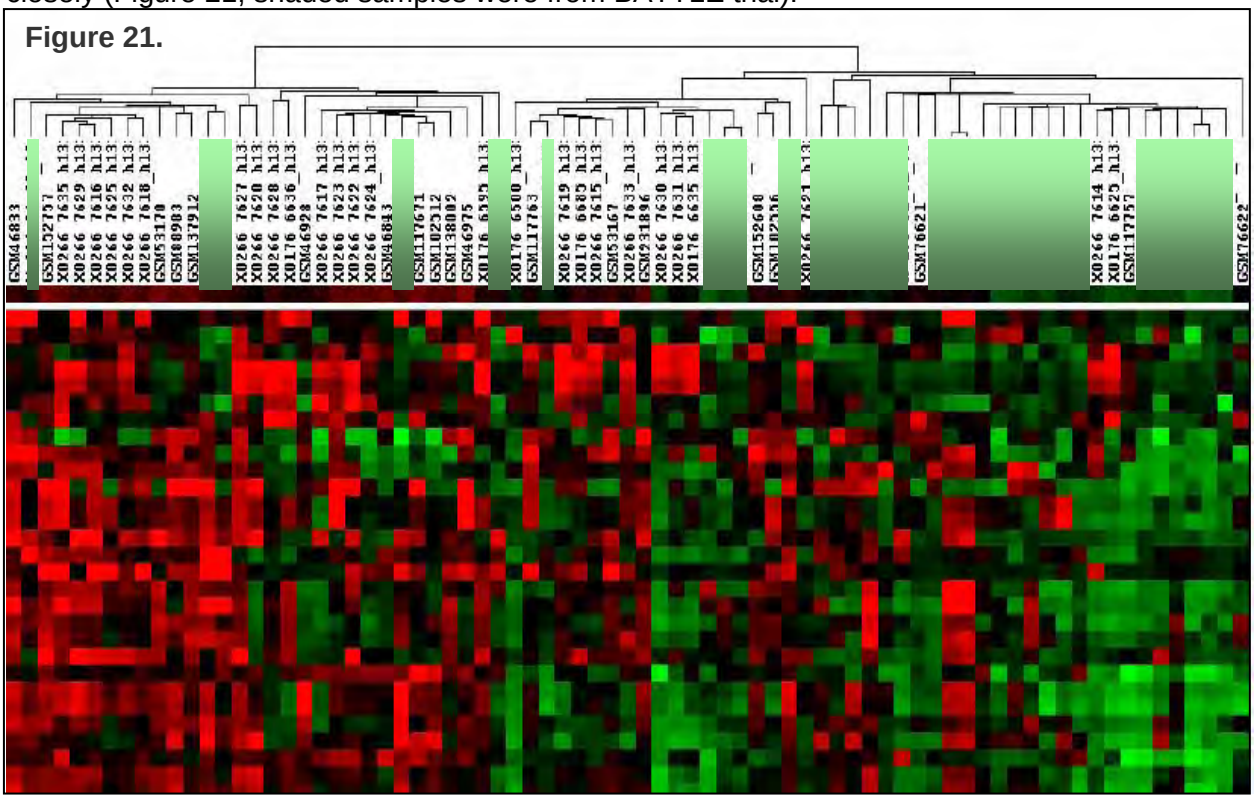
Because patients' response data to individual regimens remains blinded, we will not be able to pursue this objective at this time but will initiate the study once the data becomes available.

Objective 2: Determine the effect of targeted agents in tumor tissues, and identify novel molecular mechanisms of tumor response or progression.

Forty-three (43) specimens from the ongoing BATTLE trial have used for global gene expression analysis using U133-2 plus A&B arrays (Affymetrix, Santa Clara, CA). The initial quality analysis showed that 36 (84%) of these samples produced microarrays with profiles that qualified for further analysis. Seven samples (16%) were removed from the analysis because of poor signals. The criterium for removal was 90% of the probes on microarray chip having a signal intensity of less than 200 (90th percentile < 200). Position-dependent nearest-neighbor (PDNN) models were used to process the data array CEL files. All files were analyzed using the same energy parameter file and quantile normalization file, which was generated from the Duke dataset, containing gene expression profiles from stage IIIB and IV NSCLC not treated with chemotherapy or radiation, used for further data analysis. Because the purpose of our initial study is to identify resistant signatures of first or second line treatment, it is important to have data from treatment naive samples to serve as comparison. Additional normalization was performed using the PDNN output values and median absolute deviation was calculated for each sample. The resulted values ranged from 0.24 to 0.40. The median values ranged from 5.58 to 5.82. For the combined Duke and International Genomics Consortium (IGC) samples (45 treatment naïve tumors used for comparison in our study), there were no samples excluded based on quality problems.

Unsupervised analysis was used to classify the tumors. We observed that tumors from the BATTLE trial were generally clustered together whereas treatment naïve tumors clustered more closely (Figure 21; shaded samples were from BATTLE trial).

Figure 21.



We performed pathway analysis of the differentially expressed genes using Gene Set Enrichment Analysis (GSEA) program and Ingenuity software. We are in the process of interpreting the data and selecting the key pathways for further validation and biologic experimentation.

Key Research Accomplishments:

- Performed global gene expression analysis of 43 tumors from the clinical trial and obtained high-quality profiles from 36 (84%) tumors. Preliminary analysis has been performed to identify potential treatment resistant signatures by comparing the profiles against profiles from treatment-naïve tumors with similar stages in previously published databases.

Reportable Outcomes:

In collaboration with PIs of Clinical Trials in Aim 1, Biomarker and Biostatistics Core, and PIs of Aim 2.3:

- Tran HT, Kim ES, Lee JJ, Herbst RS, Liu S, Wistuba II, Yan S, Stewart DS, Hong WK, Heymach JV. Correlation between plasma cytokine/angiogenic factors (C/AF) and pathway activation from baseline tumor biopsy specimens in patients with advanced non small cell lung cancer (NSCLC): preliminary analysis from the Biomarker-based Approaches of Targeted Therapy for Lung Cancer Elimination (BATTLE) clinical study. AACR Annual Meeting, San Diego, CA, April 2008.

Conclusions:

Global gene expression profiles can be successfully obtained using residual tumor biopsies after fulfilling required biomarker analysis. Potential resistant signatures have been identified and are ready for further testing.

In the next year, we will continue gene expression profile analyses on more tumor tissue specimens. Due to the relatively poor quality of RNA from some of the biopsies, we will adapt new RNA amplification protocols to improve the yield to obtain high-quality gene expression profiles. Possible signatures showing a strong association with chemoresistant features will be further examined for validation and biological analysis to determine potential mechanisms. We will begin constructing reverse-phase protein microarrays (RPPA) from the tissue specimens and the corresponding serum to validate some of the findings from the gene expression profiles and serum protein analysis.

Specific Aim 4: To explore new preclinical combinations and their mechanisms of action by targeting mTOR signaling and develop phase I trials to test these combinations.

(PI and Co-PIs: Fadlo Khuri, M.D., Shi-Yong Sun, Ph.D., Haian Fu, Ph.D.)

The overall objective of Aim 4 is to study the efficacy of mTOR inhibitor combination therapies that co-target mTOR and PI3K/Akt signaling. Following is a summary of our research progress for Year 2:

Objective 1: To study the efficacy of mTOR inhibitor combination therapies that co-target mTOR and PI3K/Akt signaling.

Update

In our previous report, we showed that the combination of an mTOR inhibitor and Tarceva exhibits enhanced effects on inhibiting the growth of human NSCLC cells. We further determined the effect of the combination on the growth of human lung xenografts in nude mice. As presented in Figure 21, the combination of RAD001 and Tarceva was significantly more effective than either RAD001 or Tarceva alone in inhibiting the growth of A549 xenografts by measuring the tumor sizes (Figure 22A) and weight (Figure 22B). These results confirmed our findings in cell cultures. The combination did not significantly affect mouse body weight (Figure 22C), suggesting that the combination has limited toxicity. Currently, we are investigating the mechanisms by which the combination exhibits enhanced anticancer activity. The preliminary data suggest that inhibition of eIF4E phosphorylation may be an important event in the enhanced anticancer activity of the combination.

We also examined the effect of RAD001 in combination with perifosine on the growth of A549 xenografts in nude mice. As shown in Figure 23, the enhanced anti-tumor effects were observed only after prolonged treatments with RAD001 and perifosine combination. At the last two measurements, the combination was significantly more effective than each single agent alone in inhibiting the growth of A549 xenografts (Figure 23A). By measuring the tumor weight, we found that the combination was more effective that RAD001 alone, but not reach statistically significant (Figure 23B). Both perifosine and the combination slightly decreased mouse body weight. This decrease is likely caused by perifosine, which has equal potency to the combination in decreasing mouse body weight. In summary, it appears that RAD001 and perifosine combination is not as effective as RAD001 and Tarceva combination in inhibiting the growth of lung tumors.

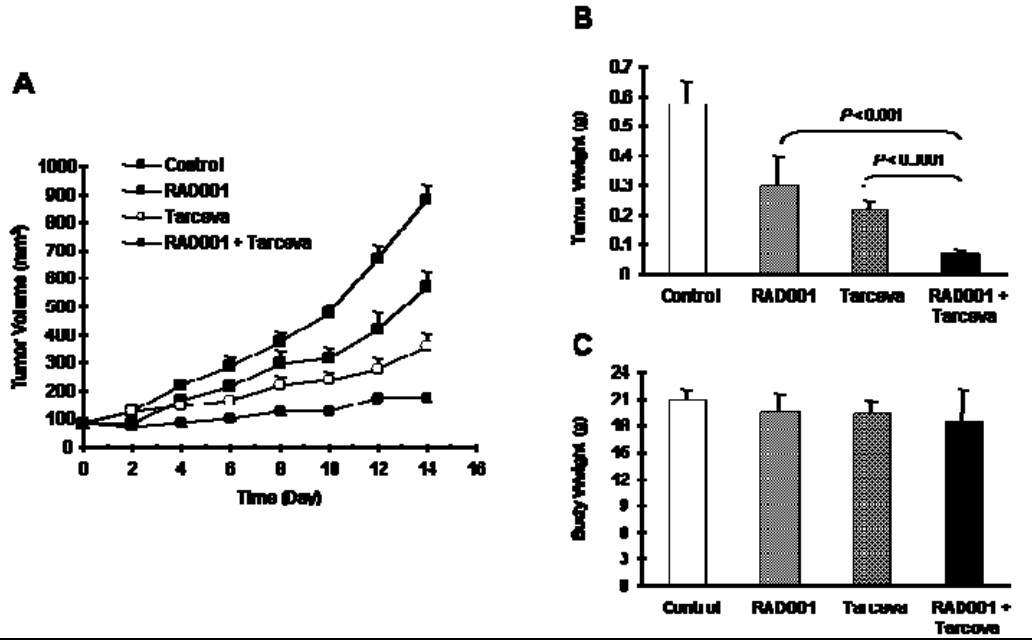


Figure 22. **Combination of RAD001 and Tarceva augments growth inhibition of lung cancer xenografts in nude mice.** Four groups of mice with A549 xenografts were treated with vehicle, RAD001 alone, Tarceva alone and RAD001 plus Tarceva on the same day after grouping. After 14 days, tumor sizes were measured once every two days as presented in **A**. At the last day, the tumor weights and mouse body weights were measured as presented in **B** and **C**, respectively. Each measurement is a mean ± SE (n=6).

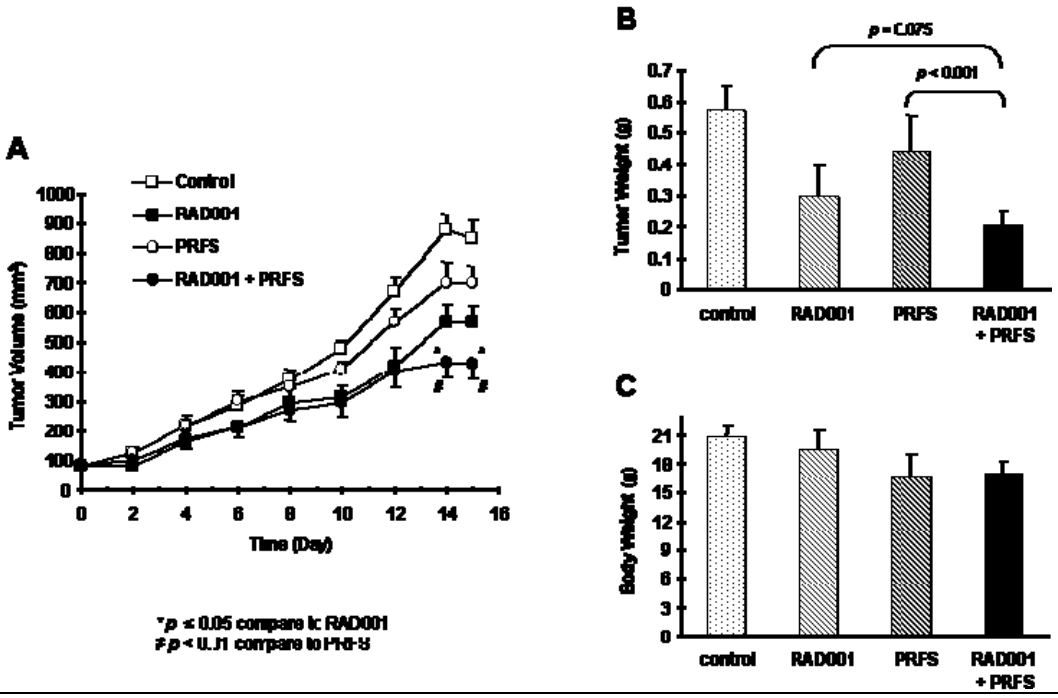


Figure 23. **Combination of RAD001 and perifosine enhances growth inhibition of lung cancer xenografts in nude mice.** Four groups of mice with A549 xenografts were treated with vehicle, RAD001 alone, perifosine alone and RAD001 plus perifosine on the same day after grouping. After 14 days, tumor sizes were measured once every two days as presented in **A**. At the last day, the tumor weights and mouse body weights were measured as presented in **B** and **C**, respectively. Each measurement is a mean ± SE (n=6).

Objective 2: To examine whether rapamycin-induced Akt activation suppresses ASK1-mediated apoptosis and leads to decreased therapeutic efficacy.

Update

Recent studies suggest that ASK1 exists in a large protein complex termed ASK1 signalosome. In order to target ASK1 signaling to enhance therapeutic efficacy in lung cancer, it is essential to

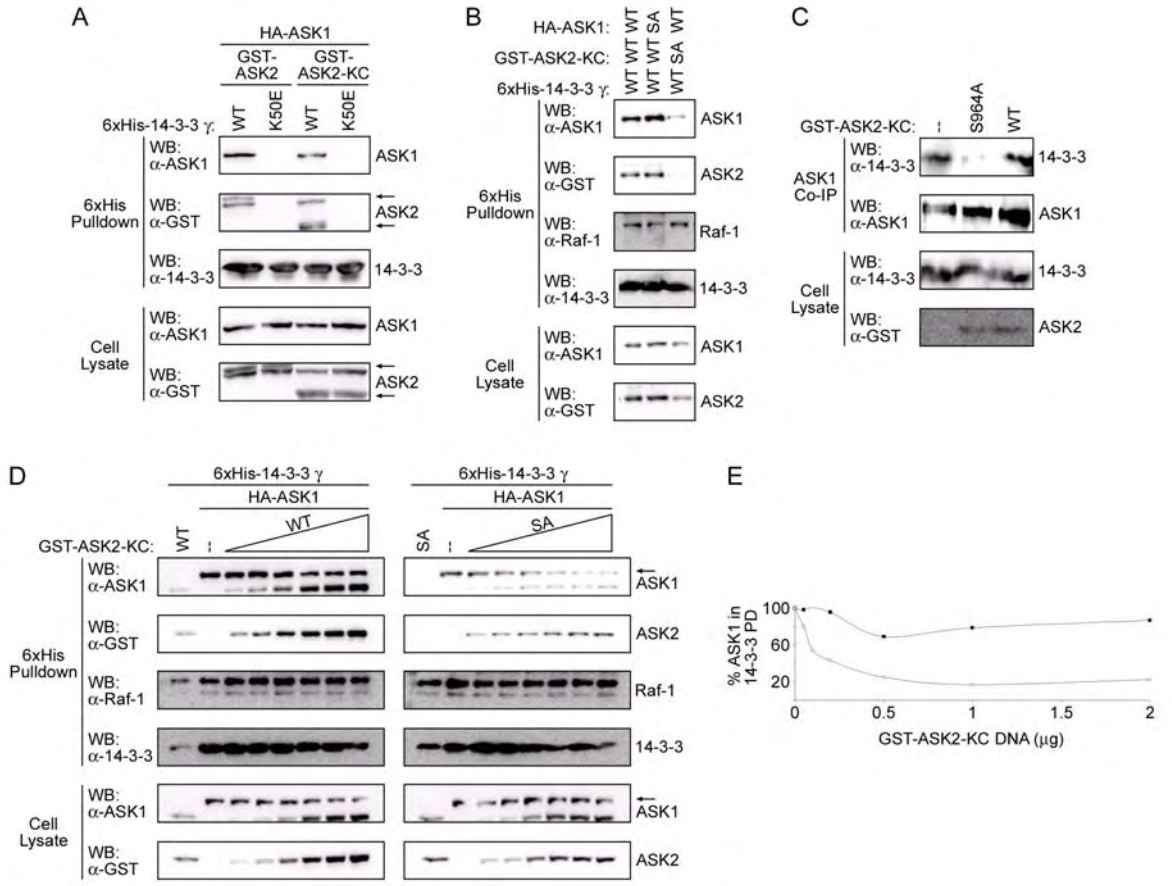


Figure 24. Association of ASK2 with ASK1 and 14-3-3 in a ternary complex. **(A)** ASK2, ASK1, and 14-3-3 exist in a multi-protein complex. HA-ASK1 and GST-ASK2 were co-transfected into Cells with either 6xHis-14-3-3 /WT or K50E. Cell lysates were used in a 14-3-3 affinity pulldown assay. The presence of HA-ASK1 and GST-ASK2 were revealed by anti-HA and anti-GST antibodies in a Western blot. **(B)** Status of ASK2 S964 controls the association of 14-3-3 with ASK1. Cells were transfected with the expression plasmids for 14-3-3, ASK1 (WT or S967A), and ASK2 (WT or S964A). 6-xHis-14-3-3 complex was isolated by affinity pull down and its associated HA-ASK1, GST-ASK2, and Raf-1 were detected with respective antibodies in a Western blot. **(C)** Mutating S964 to A in ASK2 diminishes 14-3-3 association with endogenous ASK1. HeLa cells were transfected with either pcDNA, or GST-ASK2-KC (WT or S964A). Following transfection, endogenous ASK1 was immunoprecipitated from the cell lysates using a specific anti-ASK1 antibody. Co-precipitating proteins, including the presence of endogenous 14-3-3 co-precipitated with ASK1 were detected with a pan-anti-14-3-3 antibody in a Western blot. **(D)** Increased expression of ASK2 S964A is correlated with decreased association of ASK1 from 14-3-3. COS7 cells were co-transfected with HA-ASK1 WT and 6xHis-14-3-3 along with increasing amounts of expression vectors for either GST-ASK2-KC/WT or S964A. The presence of HA-ASK1 in the 6xHis-14-3-3 complex from each sample was determined as in (B). **(E)** Quantification of data in (D). Relative fraction of ASK1 in each of 6xHis-14-3-3 pulldown sample as compared to control samples without

understand how ASK1 is controlled. We have made significant progress during the past year by

discovering a novel mechanism that dictates ASK1 signalosome function. ASK1 was shown to interact with a structurally homologous protein termed ASK2. We have found that ASK2 specifically interacts with 14-3-3 proteins in a phosphorylation-dependent manner, which is similar to how ASK1 interacts with 14-3-3. Mutational analysis revealed S964 of ASK2 as a critical site for 14-3-3 association. While a 14-3-3-binding defective mutant of ASK1 (S967A) showed no effect on the ASK2/14-3-3 interaction, over-expression of a 14-3-3 binding defective mutant of ASK2 (S964A) dramatically reduced the amount of ASK1 found in complex with 14-3-3. These data suggest a dominant role of the ASK2/14-3-3 interaction in the control of ASK1 function in this ternary complex (Figure 24). Indeed, ASK2/S964A-expression-induced dissociation of 14-3-3 from ASK1 was correlated with a decrease in the phosphorylation of ASK1 at S967 and a subsequent increase of c-Jun N-terminal kinase phosphorylation, a biological readout of ASK1 activation. Our results suggest a model that upon phosphorylation at S964, ASK2 recruits 14-3-3 to the ASK2/ASK1 signalosome, and 14-3-3 in turn binds with and suppresses ASK1. Thus, the regulation at ASK2 S964 adds a new node in the signaling network coupling upstream signals to the ASK1 signalosome through dual engagement of 14-3-3. By manipulating the protein-protein, interaction interfaces in the ASK1 signalosome may offer novel approaches to enhance therapeutic efficacy.

Objective 3: To conduct two phase I clinical trials to test the efficacy of the combination of an mTOR inhibitor with an Akt or an EGFR inhibitor in advanced NSCLC patients resistant to the front and second line therapy, and assess the modulation of targeted biomarkers from tumor tissues before and after the treatment.

Update

The Phase I trial of erlotinib and RAD001 has been conducted by Novartis based on our preclinical data and has found an MTD of 5 mg of RAD001 and 150 mg of Erlotinib. We will be participating in a Phase II trial combining RAD001 with erlotinib that is scheduled to start at Emory on July 1st. The rationale and aims of the protocol are outlined further below.

Combination of RAD001 with EGFR inhibitors

As described previously, there is evidence that targeting mTOR as well as ErbB receptors may lead to more profound effects on tumor cell biology than could be achieved through targeting the individual pathways alone. In a preliminary report (Di Cosimo 2004a, Di Cosimo 2004b), inhibition of the *in-vitro* proliferation of gefitinib (Iressa®; anti-EGFR inhibitor)-sensitive tumor cell lines (BT-474 breast and DU-145 prostate) by RAD001 was shown to be significantly enhanced when concomitantly exposed to gefitinib. This was also the case for the MDA-MB-468 breast cancer cell line, even though this line is "relatively resistant" to gefitinib. In this specific experimental setting a scheduling effect existed inasmuch as gefitinib preceding RAD001 was enhancing, whereas the opposite (exposure to RAD001 preceding exposure to gefitinib) was antagonistic. Superiority of the combination of RAD001 and the dual ErbB/VEGFR small molecule tyrosine kinase inhibitor NVP-AEE788-NX-7 compared to either agent alone also was demonstrated in an s.c lung xenograft mouse model (NCI-H596). Importantly, no evidence of antagonism of anti-tumor effect or reduction in tolerability was observed [Investigator Brochure - Doc 37, Report RD-2003-02803]. These data suggest that RAD001 is a promising candidate for use in combination with EGFR TK inhibitors in either EGFR TK inhibitor-sensitive, or resistant, tumors.

Clinical background information
In oncology, clinical experience with RAD001 is based on ongoing phase 1 studies of single-agent RAD001 and phase 1b studies of RAD001 in combination with systemic anticancer agents (imatinib, paclitaxel, gemcitabine and letrozole). Clinical experience for indications other than oncology comes from single-dose studies in healthy volunteers, studies in solid organ transplant patients, where RAD001 is co-administered with other immunosuppressive drugs, and a study in patients with severe rheumatoid arthritis.

Clinical pharmacology of RAD001
RAD001 is administered orally, its bioavailability being estimated at approximately 11%. Absorption is intestinal and delayed moderately by food (60% reduction in C_{max} and 16% reduction in AUC when drug administration follows a fat-rich meal). The AUC is consistently dose-linear with moderate inter-patient variability (CV approx 50%). C_{max} is dose-linear until 20mg increasing in a non-linear manner thereafter. The terminal half-life is 30-35 hours.

The main elimination route for RAD001 is by hepatic metabolism, mainly hydroxylation. The parent compound is the major component in the blood. Metabolism to rapamycin is of minor importance. The main metabolites are not bioactive. Excretion is (>80%) through the bile and the intestinal tract.

RAD001 is a substrate of the CYP3A4 isoenzyme and P-gp. Its metabolism is sensitive to 3A4 inhibitors/inducers. In combination with microemulsion cyclosporin A (Neoral®), a 3A4 and P-gp inhibitor, the bioavailability of RAD001 is significantly increased: for AUC by a mean of 168% (range: 46-365%); for C_{max} by 82% (range: 25-158%). RAD001 itself does not appear to have an enzyme-inducing/inhibiting effect at the levels achieved in solid organ transplantation and current oncology studies.

Mild to moderate hepatic impairment will increase exposure to RAD001. Caucasians and Japanese patients appear to be similar as regards clearance, while clearance in blacks is approximately 20% higher.

Renal function has only marginal influence on the clearance of RAD001 so that no dosage adjustment is necessary in patients with renal failure.

Clinical studies in oncology
Since 2002, RAD001 has been in phase 1 in cancer patients both as monotherapy but also in combination with a number of other anticancer agents [RAD001C Investigators' Brochure]. Phase 1 clinical studies of RAD001 as a single agent are ongoing and explore two regimens: weekly dosing (range 5-70mg) and daily dosing (5-10mg). Preliminary data are available for 84 patients, 74 advanced cancer patients, and 10 patients with newly-diagnosed prostate carcinoma. At the weekly schedule, dose-limiting toxicity (DLT) in the first four weeks of treatment has been observed at 50mg (1/12) and 70 mg (3/14). At the daily dosage, DLT has been observed at 10mg only (1/12). DLT was principally grade 3 stomatitis, but also grade 3 fatigue and neutropenia.

Apparent adverse drug reactions (ADR) include mild-moderate rash (approx. 40%), stomatitis/mucositis and fatigue (30% each), headache (20%), nausea, vomiting, diarrhea (10% each).

Reduced blood cell counts at the initiation of treatment are frequent but remain mostly within normal range, or limited to grade 1, although a grade 3 neutropenia was dose-limiting in one

patient (as was a grade 3 thrombocytopenia in a patient receiving RAD001 with letrozole where a pharmacodynamic interaction is unlikely). This suggests that some patients may be particularly sensitive to the myelosuppressive effect of RAD001 making it necessary to monitor carefully blood cell counts at initiation of treatment.

Hyperlipidemia has been reported as ADR in 10% of patients although review of the laboratory values suggests that as many a quarter of patients develop grade 1-2 hyperlipidemia on treatment, mostly hypercholesterolemia.

Infectious episodes have not been more frequent than might have been expected. Herpes infections (zoster, labialis), observed in 5 patients on the monotherapy were not severe.

Pharmacokinetic-pharmacodynamic modelling based on inhibition of a peripheral molecular marker (S6 kinase 1 activity in peripheral blood mononuclear cells) suggests that 5-10 mg daily should be adequate a dose to produce a high-degree of sustained target inhibition. Pharmacodynamic studies are underway to investigate this by investigating changes in the molecular pathology of biopsied tumor by immunohistochemistry in treated patients. Preliminary indications from these studies are that treatment with RAD001 at 5 and 10mg daily is associated with dephosphorylation of protein effectors known to be immediately downstream of mTOR. This inhibition is nearly-total in patients with phosphorylation S6, partial for 4EBP1, a picture similar to that observed preclinically in in-vivo models in which RAD001 demonstrated clear anti-tumor activity. Further analysis is ongoing and this is expected to have been completed before the end of 2004.

Experience with NSCLC
In ongoing Phase 1 studies with RAD001 monotherapy, 9 patients with advanced NSCLC have been treated as of September 2004 with weekly doses of RAD001 of 5 - 70 mg. One patient achieved a partial response (RECIST) and 3 patients had prolonged stable disease (8 weeks, 16 weeks, and 16 weeks) out of total of 6 evaluable patients failing previous 2 or more systemic therapies for advanced NSCLC.

In addition clinical responses have been reported in NSCLC patients treated with a rapamycin analog, CCI779 (Wyeth Research) (Hidalgo 2000, Forouzesh 2002) or AP23537 (Ariad Pharmaceuticals) (Mita et al 2004).

Rationale for clinical investigation of combination of RAD001 (everolimus) and erlotinib (Tarceva).
In summary, the rationale for combination of RAD001 and EGFR inhibitors in advanced NSCLC is based on following:

- Limited efficacy of EGFR inhibitors in advanced NSCLC and the medical need for better therapy for advanced NSCLC.
- Postulated association of relevant cell-signaling pathways targeted by EGFR TK inhibitors and RAD001 with different aspects of oncogenesis, disease progression, and response/resistance to treatment.
- Increased efficacy of combinations of EGFR and mTOR inhibitors in preclinical *in vitro* and in vivo experiments as compared to single agents.
- Early reports of clinical responses to monotherapy with mTOR inhibitors in advanced NSCLC.

As described above, there is evidence that an enhanced PI3K/Akt/mTOR pathway may be one of the key adaptive changes accounting for EGFR inhibitor-resistant growth in NSCLC cancer. Inhibition of this pathway might therefore prevent or delay the onset of resistance to anti-EGFR therapy. The combination of the mTOR inhibitor RAD001 and the EGFR-TK inhibitor erlotinib in NSCLC would be a novel therapeutic approach that proposes to logically manipulate the cell's regulatory pathways to enable control of tumor growth. We hypothesize that the ability to inhibit the downstream effects of Akt through inhibition of mTOR will enhance the effectiveness of the EGFR-TK inhibitors in patients with advanced NSCLC.

Potential for pharmacologic interaction between RAD001 and erlotinib other than for efficacy.

Pharmacokinetic interaction between erlotinib and RAD001 is possible as both drugs undergo CYP3A-mediated metabolism and hepato-intestinal elimination. As a result an increased exposure to one or both agents may occur. In addition, both erlotinib and RAD001 are substrates for P-glycoprotein (P-gp)-like mediated efflux systems. No preclinical experiments have been conducted evaluating PK interaction of RAD001 and erlotinib.

An additive effect might occur for those side effects characteristic of both drugs: Gastrointestinal (GI) events (diarrhea, nausea, vomiting), skin/mucosa (rash, stomatitis/mucositis) and fatigue. Adverse reactions of mild to moderate severity, which have been observed with either drug alone, could be more severe and/or frequent because of pharmacodynamic or pharmacokineitc interaction.

Phase I enrollment status and main safety observations

Since July 2005 over 40 patients have been enrolled and treated in the phase 1 part of the study evaluating the combination of once daily erlotinib combined with either once daily or once weekly RAD001.

Table 3 summarizes the status of the phase 1 part of the study as of August 1, 2007. Main adverse events observed so far were as expected from known safety profiles of erlotinib and RAD001.

Table 3. Summary of the phase 1 part study status as of 1 Aug 2007

Cohort	Erlotinib dose	RAD001 dose	Number of patients enrolled	Number of patients with DLT	Type of DLT, CTC Grade
1	100 mg,qd	5 mg, qd	6	3	3 x Stomatitis, G3
2	100 mg, qd	5 mq, q2d	12	4	Rash, G3 Stomatitis, G3 2 x Diarrhea, G3
3	150 mg, qd	30 mg, qw	6	1	Rash, G3
4	100 mg, qd	2.5 mg, qd	12	3	Rash, G3 Stomatitis, G3 Neutropenia, G3
5	150 mg, qd	50 mg, qw	12	1	Rash, G3
6	150 mg, qd	2.5 mg, qd	0	-	-
7	75 mg, qd	5 mg, qd	0	-	-

qd: once daily; q2d: once every 2 days; qw/ once weekly
CTC Grade: NIH-NCI Common Terminology Criteria for Adverse Events

Study objectives:

Primary objectives

Overall, this study aims to assess the value of combined treatment with RAD001 and erlotinib in patients with advanced NSCLC previously treated only with chemotherapy as systemic therapy. The study consists of two consecutive phases, the primary aims of which are:

In phase 1

To assess the feasibility (in terms of both dose and schedule) of combined daily or weekly administration of RAD001 with daily erlotinib based on evaluation of safety and PK drug-drug interaction and to establish the appropriate doses to carry forward into phase 2.

In phase 2

To estimate the Disease Control Rate at 3 months (DCR at 3 months) as measure of anti-tumor activity in patients who receive RAD001 (daily and/or weekly schedule) together with daily erlotinib as compared to the DCR at 3 months in patients who receive erlotinib alone.

Secondary objectives

In phase 1:

To assess the clinical efficacy of RAD001 and erlotinib combination schedule(s), based on evaluation of ORR (Objective Response Rate) and EPR (Early Progression Rate)

In phase 2:

- To describe the clinical efficacy of all study treatments in terms of ORR, PFS (Progression Free Survival) and survival
- To describe the safety profile of all study treatments
- To assess steady state drug levels
- To investigate potential molecular markers
- To evaluate tumor metabolic response with FDG-PET imaging

Overall study design

This is a combined phase 1 and phase 2 study. Phase 1 is an open label, non-randomized, multi-center study combining daily and weekly RAD001 with daily erlotinib in patients with advanced NSCLC previously treated only with chemotherapy. Phase 2 is an open label, randomized, multi-center and parallel group study assessing the relative safety and efficacy of the combined treatment of RAD001 and erlotinib combination schedule(s) versus that of erlotinib alone in the same patient population as phase 1.

Key Research Accomplishments:

- The combination of RAD001 and erlotinib or perifosine exerts enhanced anti-tumor effects in lung cancer xenografts in nude mice.

Reportable Outcomes:

None

Conclusions:

Our data show that the combination of RAD001 and Tarceva or perifosine exerts enhanced anti-tumor effects in lung cancer xenografts in nude mice, thus providing preclinical support for enhancing mTOR-targeted therapy of lung cancer in the clinic.

Biostatistics and Data Management Core

(Core Director: J Jack Lee, Ph.D.)

In close collaboration with the Biomarker Core, the clinical research team, and each of the basic science research components, the Biostatistics and Data Management Core (BDMC) for the Department of Defense (DoD) BATTLE lung cancer research program is a comprehensive, multi-lateral resource for designing clinical and basic science experiments; developing and applying innovative statistical methodology, data acquisition and management, and statistical analysis; and publishing translational research generated by this research proposal.

The main objectives of the BDMC are as follows:

1) Develop and implement a novel adaptive randomization scheme for assigning patients into the treatment arms with the highest probability of success.
2) Provide the statistical design, sample size, and power calculations for each project.
3) Develop a secure, internet-driven, web-based database network between UTMDACC and other research centers, including Emory University and the Dana-Farber Cancer Center, that integrates the clinical data generated by the five proposed clinical trials and relating basic science research efforts of the BATTLE research project.
4) Develop a comprehensive, web-based database management system for tissue specimen tracking and distribution and for a central repository of all biomarker data.
5) Provide all statistical data analyses, including descriptive analysis, hypothesis testing, estimation, and modeling of prospectively generated data.
6) Provide prospective collection, entry, quality control, and integration of data for the basic science, pre-clinical, and clinical studies in the BATTLE grant.
7) Provide study monitoring and conduct that ensures patient safety by timely reporting of toxicity and interim analysis results to various institutional review boards (IRBs), the UTMDACC data monitoring committee, the DoD, and other regulatory agencies.
8) Generate statistical reports for all projects.
9) Collaborate with all project investigators and assist them in publishing scientific results.
10) Develop and adapt innovative statistical methods pertinent to biomarker-integrated translational lung cancer studies.

Update

In the second funding year, the BDMC continued to work with all project investigators in providing biostatistics and data management support. The accomplishments are summarized below.

(A) Biostatistics

We have implemented a novel study design incorporating hierarchical Bayes model and adaptive randomization to identify the best treatment for each patient's biomarker profile and adaptively randomize more patients into more effective treatments accordingly. We have worked with clinical investigators in providing the biostatistical support in the protocol revision of the five protocols (one umbrella protocol and four treatment protocols). We provide a statistical report in our monthly project meetings to update the accrual, randomization, demographic data, etc. We have switched from the equal randomization phase to the adaptive randomization phase earlier this year. The adaptive randomization program was written in a statistical package

"R" and has been thoroughly tested. Web services were applied to integrate the adaptive randomization program with the main web-interfaced database application. The study has a steady accrual and is moving along very well.

(B) Data Management

Database task effort

1. Develop user security to allow read/write or read only access to specific parts of the BATTLE database application.
2. Apply a consistent user interface throughout the application.
3. Design a user-friendly screen/CRF navigation on left side of application.
4. Adhere to HIPAA regulations for patient confidentiality.
5. Support multiple consents as required by the protocol.
6. Inclusion and Exclusion criteria questions are presented in an easy to use, consistent format.
7. Biomarker results are displayed when Biomarker information is entered as well as Marker Group Assignment for Randomization.
8. Randomization screen displays Consent information to help maintain an easy work flow.
9. Randomization is disabled until the patient has signed the consent, biomarker entry and eligibility information has been entered.
10. Support for two type of randomization, "balanced randomization" until we have enough marker groups and treatments followed by "adaptive Randomization." An ability to do randomization when biomarker information is not available and without affecting the adaptive randomization also exists.
11. The Randomization screen also has consent for treatment information.
12. Medical history has a large area for all medical history related information. Various grids are displayed and can be easily expanded to allow for as many entries as needed by the nurse.
13. Physical Exam, Lab Tests, Study Drug Compliance Calculation, On Study EKGs and other CRF's are cycle based. These allow for similar screens to be entered for every cycle. In special cases, information can also be entered in between cycles if needed.
14. Lab Tests screen supports Urinary Analysis Not Done to avoid data entry confusion.
15. Eight Week response saves the response information so that Adaptive Randomization can use this to help ensure the patient is assigned to the most effective treatment.
16. Tumor measurement entry allows for as many lesions as needed.
17. Support for general comments can be entered as needed for every patient.
18. Biopsy Block and Slide tracking support as well as support for prior biopsies is currently being developed.
19. Support for additional clinical information is also in progress.
20. A six-week response report was added to show ongoing progress and current expectations for the head nurse.
21. Adaptive randomization testing tools are inplace to allow ongoing testing for current and prior versions of the Adaptive Randomization 'R' code.
22. Multi-level Blood sample tracking support is being evaluated for future development.
23. The Response screen now allows for inevaluable responses to be entered into the system.

Database programming effort for the BATTLE project is described as follows.

1. The C# (C Sharp) ASP 2.0 Application is running on an IIS web server using SSL for additional security and encryption. Microsoft SQL Server 2005 is used to store the data as the User/Login information.

2. The BATTLE system uses Attributes to store study related information. These attributes have unique ID's and are grouped by form so that they are easily retrieved when a specific form is brought up for a specific patient. Having these attributes available allow for quick form design, form enhancement and organized data retrieval.

3. All CRF information is stored in Attributes in the DMI_EAV_Production Database based on Form Name, Patient ID Cycle, Attribute ID and Event ID This allows for a wide variety of combinations to meet the future growth and virtually any need that may come up in the future for the BATTLE project.

4. There are two Randomization tables stored on the SQL server that allow for both Adaptive and Non-Adaptive (Equal) Randomization storage. The Non-Adaptive Randomization table allows us to randomize patients equally in the first phase of the trial conduct and for those who with insufficient biomarker information for applying adaptive randomization.

5. Randomization routines have been written in 'R' code, which is a high-level statistical software. The BATTLE database system calls a Web Service to run these randomization routines. The parameters, matrixes and results of these randomizations are stored in randomization tables in the DMI_EAV_Production database.

6. Specimen and Tracking data is stored in the BATTLE 2005-0823 database. This is much more specialized and specifically tailored to the needs of BATTLE's current and future tracking needs. Since data for tracking is usually queried using large amounts of data, these tables designed to have fast access with little to no lag.

7. Reports are being migrated to use local reporting to provide better support for multi-institution usage.

Key Research Accomplishments:

- Developed a novel adaptive randomization design.
- Developed a secured, web-based database application to assist the study conduct.

Reportable Outcomes:

A web-based database application is developed and deployed at:
https://insidebiostat/DMI_BATTLE/Common/Login.aspx

Presentations

- Lee JJ. Design for Targeted Therapies: Statistical Considerations, 12th World Conference on Lung Cancer, Seoul, South Korea, September, 2007.
- Lee JJ. Design for Targeted Therapies in Lung Cancer: Statistical Considerations, National Cancer Policy Forum, Institute of Medicine, Washington, D.C., October, 2007
- Lee JJ, Overview of the statistical properties of adaptive randomization in the BATTLE trial. Interdisciplinary Forum CCSG Lung Cancer Program. University of Texas M. D. Anderson Cancer Center, February 2008.
- Lee JJ. Novel trial designs for targeted therapy. IASLC Targeted Therapies for the Treatment of Lung Cancer, Santa Monica, California, February, 2008.
- Lee JJ. Biostatistics and Data Management Core for The BATTLE Project. Presentation to the M. D. Anderson External Advisory Board, Houston, Texas, March 2008.

Abstracts

- Liu S, Kim ES, Zhou S, Wistuba IW, Herbst RS, Lewis J, Lee JJ. An Application of Adaptive Randomization Using Hierarchical Bayes Model in a Prospective Biomarker-Based Clinical Trial. Joint Statistical Meeting, Salt Lake City, Utah, August 2007.
- Zhou X, Kim ES, Herbst RS, Liu S, Wistuba II, Mao L, Lewis J, Lippman SM, Hong WK, Lee JJ, A clinical trial design applying Bayesian adaptive randomization for targeted therapy development in lung cancer - A step toward personalized medicine. Poster presentation in the American Society of Clinical Oncology Annual Meeting, Atlanta, Georgia, June 2007.

Publications (in Press)

Zhou X, Liu S, Kim ES, Herbst RS, Lee JJ. Bayesian Adaptive Design for Targeted Therapy Development in Lung Cancer - A Step Toward Personalized Medicine. In press, *Clinical Trials*, 2008.

Conclusions:

In collaboration with clinical investigators, research nurses, the Biomarker Core, and basic scientists, the Biostatistics and Data Management Core has continued to deliver the biostatistics and data management support as proposed.

Appendices:

Appendix C: Workflow and Database Overview.

Appendix D: Database Screen Shots.

Biomarker Core: Perform biomarker assessment to stratify patients into a particular arm of clinical trials and coordinate the distribution of clinical samples.

(Core Director: Ignacio Wistuba, M.D.)

The Biomarker Core, in close collaboration with the Biostatistics and Data Management Core, the Clinical Trial team, and Research Project Investigators, has played an important role in achieving the objectives proposed in the aims of the proposed BATTLE program by acquiring and processing lung cancer tissue specimens and performing the biomarker analysis for the stratification of patients into the clinical trials. In addition, the Core has collected and banked tissue specimens to support mechanistic studies of response or resistance to targeted agents used in the BATTLE trials.

The Biomarker Core has successfully combined standard methods of histopathology processing and assessment of lung cancer tissue specimens with more advanced tools of molecular and genetic biomarker analyses.

Objective 1: To acquire, bank, process, and distribute tumor and blood specimens obtained from BATTLE enrolled patients for biomarker analyses and molecular mechanistic studies of targeted agents.

Update

The Biomarker Core collected NSCLC tumor tissue specimens from 148 patients (cases) enrolled in the BATTLE clinical trials. By March 31, 2008, NSCLC tumor core biopsies from 147 cases have been processed (formalin-fixed and paraffin-embedded) and histopathologically evaluated. Of those, 122 (83%) cases yielded enough tumor cells to examine and report a complete set of biomarkers as proposed (see Objective 2 - Table 3). In 25 (17%) patients, no viable tumor cells were detected for biomarker analysis, in which the most frequent findings were necrotic tumor tissue and dense fibrosis. Sixty-four (58%) tissue specimens were obtained from lung sites and the rest from metastasic sites (including 15 from lymph nodes, 10 from adrenal glands, 9 from liver, 7 from soft tissues, and 4 from bone). NSCLC histology types included adeno-carcinoma (61%), squamous cell carcinoma (14%), NSCLC no type otherwise specified (23%), and undifferentiated NSCLC (2%). At least one fresh tumor tissue core was snap-frozen in 132 cases, and those specimens were banked in our laboratory tissue bank facility. The formalin-fixed and paraffin-embedded (FFPE) tissue specimens for all cases are banked in the Biomarker Core, and they represent 412 tissue blocks and 3,759 unstained histology sections.

In addition, in close collaboration with the Biostatistics Core, we are collecting diagnostic, pre-chemotherapy, tissue specimens from the BATTLE patients to compare molecular markers before and after chemotherapy treatments the patients receive.

Objective 2: To perform biomarker analyses and report results in a timely fashion for patient stratification in the BATTLE trials and molecular mechanistic studies of the targeted agents.

Update

Tissue specimen workflow for biomarker analysis:
As of March 31, 2008, we have developed and sustained a system to collect and process tissue specimens, evaluate tissue quality for analysis, perform biomarker analysis, and report biomarker results into the web-based clinical trial database within 14 days. As stated above, we have reported the 11 biomarker results in 122 cases that were analyzable and we have not missed a deadline yet.

Briefly, this process is as follows:

1. Tissue specimen cores (2 – 3 cores) are collected from Interventional Radiology by Biomarker Core personnel.
2. Immediately, the specimens are transported to the lab for formalin fixation (1 or 2 cores) and snap-freezing (1 core).
3. Within 24 hours, histology sections are reviewed by a pathologist to assess tissue quality and to select areas for microdissection (DNA extraction for mutation analyses) and to prepare slides for fluorescence in situ hybridization (FISH; for EGFR and Cyclin D1) and immunohistochemistry (IHC) (6 markers).
4. If the tissue specimen is suitable for biomarker analysis, FISH, IHC and mutation analyses will be performed in the next 6-7 days. If the tissue specimen is inadequate for biomarker examination (including frozen specimens), this information is reported immediately to the clinical trial personnel.
5. IHC and FISH biomarker analyses are performed using external and/or internal controls and mutational analyses are performed in duplicate.
6. After the biomarker analysis is completed by the Biomarker Core lab personnel, two pathologists (Drs. Ximing Tang and Ignacio Wistuba, Biomarker Core Director) review independently the biomarker results and report them using the web-based electronic report.

Biomarker analyses:
The biomarkers routinely examined in the lung tumor tissue specimens are listed in Table 4. All biomarkers are examined using the formalin-fixed and paraffin-embedded tissues. DNA for mutation analyses of *EGFR*, *K-RAS* and *B-RAF* genes is extracted from microdissected tissue obtained under direct microscope observation from hematoxylin-eosin (H&E) stained tissue sections.

Table 4. Biomarkers examined in lung cancer biopsy samples and for patient stratification in clinical trials.

Molecular Pathway	Biomarkers	Type of Analysis
EGFR	*EGFR* Mutation (exons 18 to 21) *EGFR* Increased Copy Number (polysomy/amplification)	DNA sequencing DNA FISH[1]
K-Ras/B-Raf	*K-RAS* Mutation (codons 12,13, 61) *B-RAF* Mutations (exons 11 and 15)	DNA sequencing DNA sequencing
Angiogenesis	VEGF Expression VEGFR-2 Expression	Protein IHC[2] Protein IHC
RXRs/Cyclin D1	RXR α, β, γ Expression Cyclin D1 Expression Cyclin D1 Amplification	Protein IHC Protein IHC DNA FISH

[1]FISH, Fluorescent *In Situ* Hybridization. [2]IHC, Immunohistochemistry.

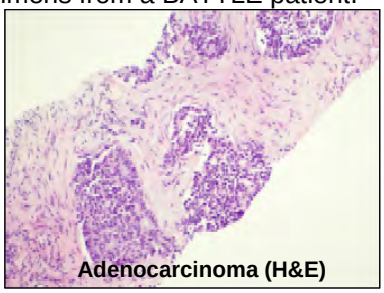
Summary of biomarkers data:

As of March 31, 2008, the complete set of biomarker results has been reported on 122 patients enrolled in the clinical trial. Data are summarized on Table 5, and a representative example of biomarker analysis from lung cancer tissue core specimens from a BATTLE patient is shown in Figure 25.

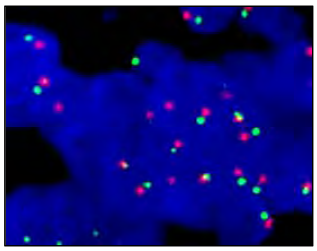
Table 5. Summary of biomarker results in 33 NSCLC cases.

Biomarker Group	**Positive Cases**
EGFR markers	72 (59%)
EGFR mutation (exons 18-21)	23 (19%)
EGFR FISH increased copy number	72 (59%)
High polysomy	41 (34%)
Gene amplification	30 (25%)
K-Ras/B-Raf	16 (13%)
KRAS mutation (codons 12, 13 and 61)	15 (12%)
BRAF mutation (exons 11 and 15)	1 (1%)
Angiogenesis	92 (77%)
VEGFR IHC expression (score >100)	90 (74%)
VEGFR-2 IHC expression (score >100)	50 (41%)
RXRs/CyclinD1	105 (86%)
RXR α nuclear IHC expression (score >30)	98 (80%)
RXR α cytoplasmic IHC expression (score >200)	1 (1%)
RXR β cytoplasmic IHC expression (score >200)	8 (7%)
RXR β membrane IHC expression (score >200)	0
RXR γ cytoplasmic IHC expression (score >200)	10 (8%)
Cyclin D1 IHC Expression (score >10%)	68 (56%)
Cyclin D1 FISH amplification	19 (16%)

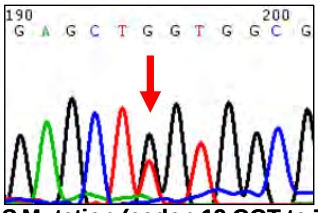
Figure 25. A representative example of biomarker analysis from lung cancer tissue core specimens from a BATTLE patient.



Adenocarcinoma (H&E)

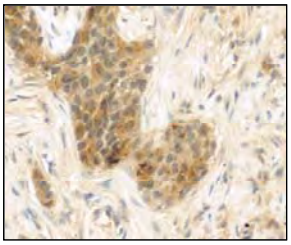


FISH - *EGFR* Low Polysomy

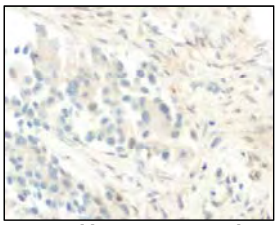


KRAS Mutation (codon 12 GGT to TGT)

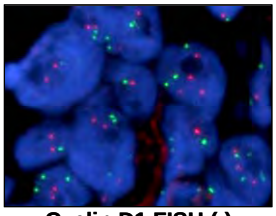
Report	
EGFR Mutation	Negative
EGFR FISH	Negative
KRAS/BRAF Mutation	Positive
VEGF/VEGFR IHC	Positive
RXR	Negative
Cyclin D	Negative



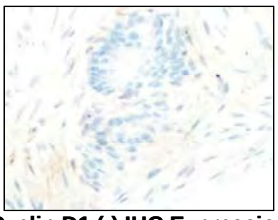
VEGF (+) IHC Expression



VEGF (-) IHC Expression



Cyclin D1 FISH (-)



Cyclin D1 (-) IHC Expression

Formalin-fixed and paraffin-embedded histology sections and fresh frozen tissues from all cases were banked for future distribution to the BATTLE research investigators for their research activities.

Key Research Accomplishments:

- Establish and maintain a system for tissue processing and molecular biomarker analysis of core biopsy tissue specimens from lung cancer patients in timely fashion (14 days).
- Collect, process and report tissue histology in all whose biopsies have been obtained
- Perform the complete set of biomarkers on all patients with suitable tissues within 2 weeks, which is vital to the success of our clinical trials.

Reportable Outcomes:

In collaboration with PIs of Clinical Trials in Aim 1, Biostatistics Core, and PIs of Aim 2.3 and 3:

- Tran HT, Kim ES, Lee JJ, Herbst RS, Liu S, Wistuba II, Yan S, Stewart DS, Hong WK, Heymach JV. Correlation between plasma cytokine/angiogenic factors (C/AF) and pathway activation from baseline tumor biopsy specimens in patients with advanced non small cell lung cancer (NSCLC): preliminary analysis from the Biomarker-based Approaches of Targeted Therapy for Lung Cancer Elimination (BATTLE) clinical study. AACR Annual Meeting, San Diego, CA, April 2008.

Conclusions:

The Biomarker Core has successfully combined standard methods of histopathology processing and assessment of lung cancer tissue samples with more advanced tools of molecular and genetic biomarker analyses for prospectively examining molecular biomarkers for individualized targeted therapy in a large number of NSCLC patients. Our next plan is to compare the pathology and biomarker expression of diagnostic tumor tissue specimens before chemotherapy with BATTLE tissue specimens and to validate predictive biomarkers identified in the profiling work currently being performed by Dr. Li Mao's Lab.

KEY RESEARCH ACCOMPLISHMENTS

Specific Aim 1: To establish a clinical trial program using biomarker assessment to select individualized targeted therapy for previously treated chemorefractory advanced NSCLC patients.

- Activated all five BATTLE clinical protocols at M. D. Anderson Cancer Center within the first grant year.
- 166 patients registered and 119 randomized into one of the four treatment arms.
- Adaptive randomization phase accruing.
- Patient accrual and interest continue at a healthy pace.
- The success rate of quality tissue specimen acquisition and biomarker evaluation is over ~84%.
- Demonstrated highly efficient collaboration of Clinical teams, Biostatistics Core, and Biomarker Core.
- Developed the largest translational research program requiring core biopsy samples ever run in our department and, possibly, in the country.

Specific Aim 2.1. To validate the molecular mechanisms of response and resistance to erlotinib for patients with chemorefractory NSCLC.

- Thirty-five (35) patients participating in the BATTLE program have been allocated to the trial entitled, "A Phase II, Open Label Study of Erlotinib (Tarceva®) in Previously Treated Subjects with Advanced Non-Small Cell Lung Cancer."
- Amphiregulin is the agonist in tumor cell lines (both lung cancer and head and neck cancer cell lines) with wild type EGFR that is associated with response to gefitinib and cetuximab.

Specific Aim 2.2. To investigate whether the resistance to erlotinib is mediated by the activation of type I insulin-like growth factor receptor (IGF-1R) signaling pathway

- Gefitinib inhibits HSCLC cell proliferation by inducing apoptosis when IGF-IR signalling was suppressed.
- Treatment with gefitinib, but not cetuximab, induced EGFR: IGF-IR interaction and activation of EGF-IR and its downstream signalling mediators, resulting in increased survivin expression in NSCLC cell lines with a high level of IGF-IR expression.
- Inhibition of IGF-IR activation and knockdown of survivin expression led to increased apoptosis.
- Overexpression of survivin protected cells with low IGF-IR expression from gefitinib-induced apoptosis. Most NSCLC tissues with EGFR overexpression had associated high levels of IGF-IR expression.

Specific Aim 2.3. To investigate the molecular mechanisms of resistance to and biomarkers of the biologic activity of inhibitors of the VEGF pathway

- Successfully collected between 77% to 100% (91% overall) of potential samples (consent is optional) at various time-points during course of treatment.
- Measured and analyzed the first group of baseline plasma samples using multiplex bead-based and ELISA assays.

Specific Aim 2.4. To investigate the molecular mechanisms of the effects of the combination of bexarotene and erlotinib on NSCLC cells

- The level of the nuclear RXRα and the cytoplasmic RXRβ and RXRγ protein decreases across the different pathological stages of NSCLC; higher TN stage had lower level of RXRs.
- The level of the nuclear RXRα and the cytoplasmic RXRβ (in adenocarcinomas) and RXRγ protein (in SCCs) decreases with tobacco exposure.
- The sensitivity to growth inhibition by Tarceva of more progressed cells (transformed and tumorigenic) with elevated expression of EGFR was not higher than that of immortalized cell lines.
- Bexarotene can induce the tumor suppressor GPRC5A in human lung cancer cells.
- Cell lines that expressed higher levels of GPRC5A also expressed higher levels of EGFR and GPRC5A is phosphorylated in cells treated with EGF.
- The novel RXR agonist 9cUAB76 is much more potent in growth inhibition of premalignant lung cells than a RARβ agonist (9cUAB30Amide). The marked growth inhibition observed already after 1 day of treatment with the RXR agonist suggests that this rexinoid induces cell death.

Specific Aim 3: To identify biomarkers as novel predictors of clinical end points and potential therapeutic targets

- Performed global gene expression analysis of 43 tumors from the clinical trial and obtained high-quality profiles from 36 (84%) tumors. Preliminary analysis has been performed to identify potential treatment resistant signatures by comparing the profiles against profiles from treatment-naïve tumors with similar stages in previously published databases.

Specific Aim 4: To explore new preclinical combinations and their mechanisms of action by targeting mTOR signaling and develop phase I trials to test these combinations.

- The combination of RAD001 and erlotinib or perifosine exerts enhanced anti-tumor effects in lung cancer xenografts in nude mice.

Biostatistics and Data Management Core:

- Developed a novel adaptive randomization design.
- Developed a secured, web-based database application to assist the study conduct.

Biomarker Core:

- Establish and maintain a system for tissue processing and molecular biomarker analysis of core biopsy tissue specimens from lung cancer patients in timely fashion (14 days).
- Collect, process and report tissue histology in all whose biopsies have been obtained
- Perform the complete set of biomarkers on all patients with suitable tissues within 2 weeks, which is vital to the success of our clinical trials.

REPORTABLE OUTCOMES:

Presentations

- Herbst R, Lee JJ. The BATTLE Project. Presentation to the M. D. Anderson National Cancer Institute Cancer Center Support Grant External Advisory Board. Houston, Texas; January 2007.
- Herbst, R. Institute of Personalized Cancer Therapy Retreat, The University of Texas M. D. Anderson Cancer Center, Houston, TX. February 2008.
- Lee JJ. Design for Targeted Therapies: Statistical Considerations, 12th World Conference on Lung Cancer, Seoul, South Korea, September, 2007.
- Lee JJ. Design for Targeted Therapies in Lung Cancer: Statistical Considerations, National Cancer Policy Forum, Institute of Medicine, Washington, D.C., October, 2007
- Lee JJ, Overview of the statistical properties of adaptive randomization in the BATTLE trial. Interdisciplinary Forum CCSG Lung Cancer Program. University of Texas M. D. Anderson Cancer Center, February 2008.
- Lee JJ. Novel trial designs for targeted therapy. IASLC Targeted Therapies for the Treatment of Lung Cancer, Santa Monica, California, February, 2008.
- Lee JJ. Biostatistics and Data Management Core for The BATTLE Project. Presentation to the M. D. Anderson External Advisory Board, Houston, Texas, March 2008.

Abstracts

- Liu S, Kim ES, Zhou X, Wistuba II, Herbst RS, Lewis J, Lee JJ. An Application of Adaptive Randomization Using Hierarchical Bayes Model in a Prospective Biomarker-Based Clinical Trial. Submitted to the Joint Statistical Meeting, Salt Lake City, Utah; August 2007.
- Tran HT, Kim ES, Lee JJ, Herbst RS, Liu S, Wistuba II, Yan S, Stewart DS, Hong WK, Heymach JV. Correlation between plasma cytokine/angiogenic factors (C/AF) and pathway activation from baseline tumor biopsy specimens in patients with advanced non small cell lung cancer (NSCLC): preliminary analysis from the Biomarker-based Approaches of Targeted Therapy for Lung Cancer Elimination (BATTLE) clinical study. AACR Annual Meeting, San Diego, CA, April 2008.
- Zhou X, Kim ES, Herbst RS, Liu S, Wistuba II, Mao L, Lewis J, Lippman SM, Hong WK, Lee JJ. A clinical trial design applying Bayesian adaptive randomization for targeted

therapy development in lung cancer - A step toward personalized medicine. Submitted to American Society of Clinical Oncology (ASCO) Annual Meeting, Atlanta, Georgia; June 2007.

Publications

Morgillo F, Woo JK, Kim ES, Ciardiello F, Hong WK, **Lee H-Y**. Implication of the Insulin-like Growth Factor-1R Pathway in the Resistance of Non-small Cell Lung Cancer Cells to Treatment with Gefitinib. Clin Cancer Res, 13(9):2795-2803, 2007.

Publications (In Press)

- Zhou X, Liu S, Kim ES, Herbst RS, Lee JJ. Bayesian Adaptive Design for Targeted Therapy Development in Lung Cancer - A Step Toward Personalized Medicine. In press, *Clinical Trials*, 2008.

Publications (Submitted)

- Yonesaka K, Zenullahu K, Lindeman N, Homes AJ, Jackman DM, Zhao F, Rogers AM, Johnson BE, Janne PA. Autocrine production of amphiregulin predicts sensitivity to both gefitinib and cetuximab in *EGFR* wild type cancers. Clinical Cancer Research, submitted.

Other

A web-based database application is developed and deployed at:
https://insidebiostat/DMI_BATTLE/Common/Login.aspx

CONCLUSIONS

Aim 1: The completion of the clinical trials is the key to this BATTLE research program. In the first grant year, the program was significantly ahead of our proposed timeline. This has continued after the first year of activation and, in addition to the trial performance, we have supported the early efforts in biomarker discovery in collaboration with the other projects. The trial accrual is reflective of the goals of the department and the program in its completion. The design and innovative nature of the trials will keep interest high among patients who are treated at M. D. Anderson. Accrual is ongoing and will help support the other BATTLE specific aims with tumor response data, tissue specimens, and biomarker information.

Aim 2.1: Amphiregulin is the agonist in tumor cell lines (both lung cancer and head and neck cancer cell lines) with wild type EGFR that is associated with response to gefitinib and cetuximab. These agonists and the determination whether the EGFR receptor is mutated will need to be studied in the tumor specimens from the patients participating in the phase II trial of erlotinib to see if these *in vitro* findings translate into the clinical specimens available from the patients participating in this study.

Aim 2.2: Overall, our findings suggest that activation of IGF-IR and consequent induction of survivin expression contribute to the acquired resistance to EGFR tyrosine kinase inhibitors (TKIs), but not to the monoclonal antibody against EGFR in NSCLC cells. Suppression of IGF-IR signaling pathways may prevent or delay development of gefitinib resistance in patients with NSCLC.

Aim 2.3: Several plasma CAFs are associated with specific tumor-derived pathway activation. This preliminary study suggests that broad-based plasma profiling of cytokines and angiogenic factors may be a feasible approach for identifying markers of activation of tumor signaling pathways. In addition to the evaluation of pathway activation using plasma samples, we will be evaluating modulation of CAFs by each treatment arm, evaluating for potential predictive plasma signature(s) with clinical outcome measures such as progression-free survival (PFS). The final step will be to validate the plasma predictive signature derived from BATTLE with other randomized clinical studies.

Aim 2.4: Altered retinoid receptors expression may play a role in the pathogenesis and progression of these tumors because various retinoid-controlled pathways, including cellular differentiation and cell cycle control depend on intact receptor function. Similarly, decreased level of RXRs in smokers may compromise their response to the RXR ligand Bexarotene in the clinical trial.

This suggests that there may be cross-talk between EGFR signaling and GPRC5A signaling and that Bexarotene may affect this cross-talk by increasing the expression of GPRC5A in cells where it is suppressed. We will pursue this mechanism in the coming year as it may be relevant to the effects of Bexarotene in the patients.

Our results indicate that the RXR agonist may be superior to RAR selective agonists against lung cancer. We will continue to explore the activity of the 9cUAB76 against a collection of NSCLC cell lines available to us.

Aim 3: Global gene expression profiles can be successfully obtained using residual tumor biopsies after fulfilling required biomarker analysis. Potential resistant signatures have been identified and are ready for further testing.

In the next year, we will continue gene expression profile analyses on more tumor tissue specimens. Due to the relatively poor quality of RNA from some of the biopsies, we will adapt new RNA amplification protocols to improve the yield to obtain high-quality gene expression profiles. Possible signatures showing a strong association with chemoresistant features will be further examined for validation and biological analysis to determine potential mechanisms. We will begin constructing reverse-phase protein microarrays (RPPA) from the tissue specimens and the corresponding serum to validate some of the findings from the gene expression profiles and serum protein analysis.

Aim 4: Our data show that the combination of RAD001 and Tarceva or perifosine exerts enhanced anti-tumor effects in lung cancer xenografts in nude mice, thus providing preclinical support for enhancing mTOR-targeted therapy of lung cancer in the clinic.

Biostatistics and Data Management Core: In collaboration with clinical investigators, research nurses, the Biomarker Core, and basic scientists, the Biostatistics and Data Management Core has continued to deliver the biostatistics and data management support as proposed.

Biomarker Core: The Biomarker Core has successfully combined standard methods of histopathology processing and assessment of lung cancer tissue samples with more advanced tools of molecular and genetic biomarker analyses for prospectively examining molecular biomarkers for individualized targeted therapy in a large number of NSCLC patients. Our next plan is to compare the pathology and biomarker expression of diagnostic tumor tissue specimens before chemotherapy with BATTLE tissue specimens and to validate predictive biomarkers identified in the profiling work currently being performed by Dr. Li Mao's Lab.

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